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TOWNSVILLE CULICINES AS POSSIBLE VECTORS OF DENGUE AND ALLIED VIRUSES AMONG LOCAL FERAL FAUNA

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(*Accepted for publication 13th November, 1959.*)

SUMMARY.

After an epidemic of dengue-like fever in Townsville in 1952, investigations were made to try to determine the possible vectors of the virus from the presumptive reservoir of flying foxes and/or migratory birds of the seashore to man. This was done by determining which mosquito species had fed upon flying foxes, upon birds, and upon man.

Two thousand and twenty female mosquitoes of 32 species were collected, either attracted to bait (man, horse and flying fox), were caught in light traps, or recovered from resting sites. Of these mosquitoes, 713 were engorged with blood and precipitin-tested to determine the hosts upon which they had fed. The range of hosts was man, horse, ox, dog and fowl.

Only five species, *T. uniformis, A. vigilax, C. annulirostris, C. fatigans* and *C. sitiens* were implicated as being possible vectors of the virus from the presumed reservoir to man, and of them the prime suspects were *A. vigilax* and *C. sitiens*, for these two species were attracted to flying fox bait, they had fed upon both man and fowl and they were associated with mangrove swamps, in which flying foxes "camp" during the day and in which migratory birds of the seashore roost at night.

These findings are discussed with regard to the implicated vectors of Murray Valley encephalitis and Japanese B encephalitis, both of which are immunologically related to dengue fever.

INTRODUCTION.

Following the outbreak of a dengue-like fever in Townsville, north Queensland, early in 1954 (Rowan, 1957), it was suggested that flying foxes (*Pteropus gouldi*) and various birds of the seashore [Silver gull (*Larus novae-hollandiae*), Redcapped dotterel (*Leucopolius alexandrinus ruficapillus*), Rednecked stint (*Callidris rufficollis minuta*), Greytailed tattler (*Heteroxcelus brevipes*) and Sharptailed sandpiper (*Callidris acuminata*)] might serve as reservoirs of the virus of dengue fever at Townsville, as antibodies to the Dengue 1, Dengue 2, Murray Valley encephalitis, Japanse B encephalitis and West Nile viruses were

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recovered from the sera of these animals (O'Connor *et al.*, 1955; Rowan and O'Connor, 1957).

Because the isolation of virus from mosquitoes is an expensive procedure which often gives negative results (Mackerras *et al.*, 1955), these investigations, which were conducted in Townsville late in 1954 and late in 1955, were designed so that the species which were probably involved in the transmission of virus from the presumed reservoir to man, could be determined by indirect means. This was done by collecting mosquitoes which inhabit areas frequented by the suspected animal reservoir of these viruses, either from their resting sites or attracted to human and animal baits and to light traps.

Methods.

Mosquitoes were collected in light traps or by aspiration from bait and from resting sites. Two light traps were operated close to human habitation from before dusk until after dawn (one with an incandescent, frosted globe of 40 watts at 240 volts A.C., the other having a twin, acetylene burner supplied with gas from an acetylene generator). The light traps were suspended close to human habitation in fruit trees, in mangrove trees and in *Melaleuca* in hyacinth swamps. The insect-holding cages of the light traps were cleared at three-hourly intervals throughout the night.

Resting sites successfully searched included outhouses and fowlsheds in the vicinity of habitation near either mangrove or hyacinth swamps, in the marginal vegetation of such areas, and amongst the fronds and stalks of the hyacinth plants. These resting sites were searched in the mornings before blood digestion could have progressed beyond the stage of identification by the precipitin test (O'Gower, 1956).

The recovery of mosquitoes in areas of low illuminance within outhouses and fowlsheds near vegetation was facilitated by using a "red light" torch. In vegetation and in the hyacinth swamps the mosquitoes were collected by first disturbing them from their rest, and then catching as many as possible "on the wing" before they either flew further away, or else rested again in the vegetation or in the hyacinth.

Man, horse and flying fox were used as bait for limited periods in this study, both near and away from the obvious breeding areas of mosquitoes. Mosquitoes attracted to such bait were collected from before dusk until activity was markedly reduced some four hours later (6 p.m. to 10 p.m.).

The collected mosquitoes were identified and those with abdomens more than half-filled with blood (O'Gower, 1956) were smeared whole (Eligh, 1952) onto filter papers. These blood smears were kept over silica gel in a refrigerator until tested against the range of antisera. The antisera[2] were prepared after the method of Proom (1943) and the details of their preparation and of the execution of the precipitin test have been reported elsewhere (Lee *et al.*, 1954). The range of antisera used was: man, horse, ox, dog, cat, domestic fowl and duck, pig, sheep, flying fox (*Pteropus*), marsupial (*Trichosurus*) and lizard (*Varanus* and *Tiliqua*). However, the antisera prepared from domestic fowl gave positive reactions to the sera of the first eight of the following twelve birds. These birds belonged to the following natural orders (Leach, 1958): Anseriformes (domestic duck and black duck), Columbiformes (dove), Passeriformes (black-faced cuckoo shrike, sparrow), Circoniformes (straw-necked ibis, Pacific heron, white-necked ibis), Charadriiformes (silver gull, red-necked stint), Falconiformes (brown hawk, whistling eagle).

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[2] The precipitin tests were executed by Mr. K. J. Clinton of the School of Public Health and Tropical Medicine, University of Sydney.

RESULTS.

The results of collections of both sexes of mosquitoes in light traps, from human and animal bait, and from resting sites are given in Table 1. However, the samples of the populations in resting habitats were unbalanced, as engorged females were sought in preference to males, and to unfed and gravid females.

Of the 32 species recovered, 11 were collected from bait, 16 were collected in light traps and 23 were collected from hyacinth swamps, and a total of 3,895 mosquitoes was collected by all techniques. *Aedes vigilax* was the only species of the 11 attracted to man which was not collected in appreciable numbers from resting sites; however, it was collected in light traps. Both *Aedes longirostris* and *Aedes tremula* were caught only in light traps. *Taeniorhynchus crassipes, Ficalbia metallica, Uranotaenia albescens, Uranotaenia nivipes, Culex fatigans, Culex pullus* and a species of the *Culex fraudatrix* complex were only collected from resting sites, and, except for *C. fatigans*, all were found resting predominantly in hyacinth swamps. Thus the three methods of collection—light trapping, animal baiting and the searching of resting sites—gave comprehensive samples of the mosquito population present in the study area, but, of these methods, the collection of adults from resting sites was the most comprehensive and efficient, both numerically and specifically (Table 1).

The mosquito recovery was generally low with a mean of 19 mosquitoes collected per hour by all techniques. The rate was highest for collections made from resting sites, in particular from the hyacinth swamp (49 mosquitoes per hour in the polluted hyacinth swamp and 40 per hour in the unpolluted hyacinth swamp). It was intermediate from baiting collections (10 per hour from man, 14 per hour from horse and 3 per hour from flying fox) and it was low from light trapping (6 per hour).

Of the total of 2,020 female mosquitoes collected by all techniques, 713 (35 p.c.) were engorged with blood, and of these the source of the blood meal was determined for 549, the remainder (164) gave negative results to precipitin testing using the available range of antisera (see Tables 1 and 2 for totals). The range of hosts upon which mosquitoes had fed was: man, horse, ox, dog and fowl and although none of the 20 species of engorged mosquitoes was found to have fed on flying fox, 12 had fed on fowl and 8 had fed on man. Of these species, *Taeniorhynchus uniformis, A. vigilax, Culex annulirostris, Culex halifaxi* and *Culex sitiens* had all fed upon both man and fowl (Table 2), whilst *A. vigilax* and *C. sitiens* were caught over flying fox bait (Table 1).

DISCUSSION.

Protective antibodies against dengue fever Type 1 were detected by the mouse protection test in the sera of five species of shore birds (Rowan and O'Connor, 1957) and against dengue fever Type 1 and Type 2, Murray Valley

TABLE 1.

Species and numbers of mosquitoes collected from bait, in light traps and from various habitats.

	Bait			Resting								Light trap						Totals	
	Man	Flying Fox	Horse	Grasses		Hyacinth polluted		Hyacinth fresh		Outhouses		Mangroves		Fruit trees		Hyacinth			
Species	F	F	F	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
An. a. amictus	2		2															4	
An. annulipes	2									2				1	2			5	2
An. b. bancroftii				1														1	
T. uniformis	45		53	5	3	98	99	19	124	1				4				225	226
T. septempunctata	1		1	1		10	3		2					4	9		1	17	15
T. crassipes					7		49		2										58
T. xanthogaster	2			3	5	10	17	2	1					14	39	2		33	62
A. catasticta														3	2			3	2
F. elegans				2	5	226	336	14	25					75	253		1	317	620
F. metallica				8	3	121	42	105	48									234	93
H. cairnensis						2												2	
U. albescens				4	5	34	16	4	11									42	32
U. nivipes				1		54	43	35	13									90	56
U. sp. No. 66												1		2				3	
A. aegypti	7										6							7	6
A. elchoensis	1																	1	
A. longirostris														21				21	
A. notoscriptus										1					3			1	3
A. purpureus	1																	1	
A. vigilax	20	3	2			1						7		59	37			92	37
A. tremula												1	4	5	15			6	19
C. annulirostris	48		26	50	15	167	67	22	9	18	7	1		21	267			453	365
C. bitaeniorhynchus						1	5	1										2	5

TABLE 1 (*Continued*).

	Bait			Resting								Light trap						Totals	
	Man	Flying Fox	Horse	Grasses		Hyacinth polluted		Hyacinth fresh		Outhouses		Mangroves		Fruit trees		Hyacinth			
Species	F	F	F	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M
C. fatigans				1		23	15			235	39	1						260	54
C. f. annulata												1			3			1	3
C. fraudatrix ssp.						18	25		2									18	27
C. halifaxi										2								2	
C. hilli						1	3											1	3
C. p. australicus														1				1	
C. pullus						39	33	1										40	33
C. sitiens	10	2	2	3	1	1	2			13	3	54	12	51	136			136	154
C. squamosus										1								1	
Totals	139	5	86	79	44	806	775	203	237	273	55	66	16	361	766	2	2	2020	1875

TABLE 2.

Results of precipitin tests upon mosquitoes engorged with blood.

Species	Site or method of collection	Origin and numbers of blood meals					Total number of engorged mosquitoes
		Man	Horse	Ox	Dog	Fowl	
An. annulipes	Fowl shed				2		2
	Man bait			1			2
An. amictus amictus	Man bait						2
T. uniformis	Hyacinth swamp		30	28		35	141
	Man bait	11	3				20
	Fowl shed					2	2
T. septempunctata	Hyacinth swamp		1				3
	Man bait						1
	Fowl shed					1	2
T. xanthogaster	Hyacinth swamp			3			3
	Light trap					2	2
F. elegans	Hyacinth swamp		15	7		26	98
	Light trap					18	22
U. albescens	Hyacinth swamp		2			1	7
U. nivipes	Hyacinth swamp			1		4	14
A. aegypti	House	7					7
A. notoscriptus	Fowl shed	1					1
A. purpureus	Man bait	1					1
A. vigilax	Hyacinth swamp	2		1		1	4
	Man bait	19					20
	Light trap					10	10
C. annulirostris	Hyacinth swamp	2	1	6		22	39
	Fowl shed	1					18
	Man bait	18		1			22
	Light trap					12	13
C. bitaeniorhynchus	Hyacinth swamp						1
C. fatigans	Fowl shed					218	222
	Hyacinth swamp			4		1	8
C. fraud[illegible] sep.	Hyacinth swamp			3			4
C. halif[illegible]	Fowl shed	1				1	2
C. hilli	Hyacinth swamp				1		3
C. sitiens	Man bait	3					3
	Fowl shed	7				3	13
C. squamosus	Fowl shed					1	1
	Totals	73	52	55	3	358	713

encephalitis, Japanese B encephalitis and West Nile fever in the sera of flying foxes (O'Connor *et al.*, 1955; Rowan and O'Connor, 1957). As some of these species of birds traverse the Far East and Australia during their annual trans-equatorial migration and as Hamilyn-Harris (1931) observed an association between the occurrence of epidemics of dengue fever in both regions, then the outbreak of dengue-like fever at Townsville in 1954 (Rowan, 1956) could have been due to the introduction of the virus by migratory birds. If so, the subsequent transmission of virus from bird to man, either directly or indirectly through flying foxes, was almost certainly by arthropod vectors, as is the case with Japanese B encephalitis (Reeves and Hammond, 1946) and presumably

also with Murray Valley encephalitis (McLean, 1953), both viruses being immunologically related to the dengue fever group (Casals and Brown, 1954; Smithburn, 1954; Sweet and Sabin, 1954).

The failure to isolate the virus of dengue fever from mosquitoes at Townsville in 1954 (Mackerras *et al.*, 1954) was possibly due to an insufficiently large reservoir of virus at the time of investigation, for the epidemic of the dengue-like fever had almost ceased, and the total number of mosquitoes collected by the party was 3,069 compared with 33,329 for a similar period in the same area but in a different season early in 1952. Therefore, any incrimination of a mosquito species as a vector of dengue fever from the presumed reservoir of birds and/or flying foxes to man, must be based upon the indirect evidence of the range of hosts bitten by different species of mosquitoes.

The composition and numbers of the various species in the mosquito population sampled at Townsville at the end of the dry season in December, 1954, indicated that the population was at a low level (Table 1). However, as the first cases of dengue fever usually occurred late in November and early in December (Rowan, 1959) and as it has been shown in allied fields that an increase in the percentage of the mosquito population, which is infected with virus, precedes by some few weeks the appearauce of infection in the human population (Sabin, 1955), then sampling of tbe low mosquito population at this time of the year should prove more fruitful in the recovery of virus than sampling higher mosquito populations at other times of the year.

The proven vector of dengue fever within the human population in Australia is *Aedes aegypti* (Bancroft, 1906; Cleland *et al.*, 1917); but, although this species was commonly encountered in Townsville, it seems unlikely that it could also be the vector of the virus from the presumed reservoir to man, as it is so closely associated with man alone.

As the antisera prepared after Proom (1943) were non-specific for the blood of so many of the birds tested, the positive precipitin reactions against fowl antiserum were taken as an indication of mosquito feeding on avian hosts. Therefore, if flying foxes and migratory birds are the reservoir of dengue fever virus and allied, arthropod-borne fever viruses, and if the mosquito population at the time of this investigation was essentially similar to that prevailing during the outbreak of the dengue-like fever earlier in 1954 (Rowan, 1957), then only five of the 32 species of mosquitoes collected in Townsville during this investigation appear to be suspect as vectors of the disease from the presumed reservoir to man, because only five species, *T. uniformis, A. vigilax, C. annulirostris, C. fatigans* and *C. sitiens* were (i) present in sufficient numbers (Table 1), and had (ii) fed upon both man and the presumed reservoir (Tables 1 and 2 and the known selection of man as a blood meal source by *C. fatigans*).

A. vigilax and *C. sitiens* both breed in brackish water, the former in open ground pools left after rain or high tides, and the latter in brackish swamps. Both

species have been found to feed upon both man and birds, the former in particular being a vicious man-biter, and both were attracted to flying fox bait. Therefore, as both species are closely associated with mangrove swamps in which flying foxes "camp" during the day and in which migratory birds of the seashore roost at night, and as both species bite freely night and day, then they must be the prime suspects as vectors of the virus from the presumed reservoir to man. *T. uniformis* and *C. annulirostris* must also be suspect as vectors of the virus for similar reasons, for, although they both breed in fresh water swamps, there are certain areas in Townsville where these swamps are close to the seashore, to mangrove swamps and to human habitation. There is also the added possibility that flying foxes may be bitten by these species when they feed upon domestically grown fruits at night. Similarly, *C. fatigans* must also be suspect, as it is so closely associated with the domestic environment in which transmission is likely to occur.

Three of these five species, *A. vigilax, C. annulirostris* and *C. fatigans,* have been found to be experimentally capable of transmitting Murray Valley encephalitis (McLean, 1953), the latter two species being amongst those suspected as vectors (Reeves *et al.,* 1954) whilst two of them, *C. annulirostris* and *C. fatigans,* have been shown to be experimental vectors of Japanese B encephalitis (Hurlbut and Thomas, 1949; Reeves and Hammond, 1946), and, as these two viruses are immunologically related to dengue fever (Casals and Brown, 1954; Smithburn, 1954; Sweet and Sabin, 1954), then *A. vigilax, C. annulirostris* and *C. fatigans* are probably capable of transmitting dengue fever. The two remaining species, *T. uniformis* and *C. sitiens,* are also probably capable of transmitting dengue fever, for other species of *Taeniorhynchus* have been shown to be naturally infected in the Americas with other similar viruses (Gilyard, 1944; Howitt *et al.,* 1949) and *C. sitiens* is definitely closely related to *C. annulirostris.* Thus, although virus was not recovered from any species of mosquitoes at Townsville (Mackerras *et al.,* 1955), the indirect evidence available as to the probable vectors of the dengue-like virus from the presumed reservoir to man indicts the following five species: *A. vigilax, C. annulirostris, C. fatigans, C. sitiens* and *T. uniformis.*

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A FLUORIMETRIC MICRODETERMINATION OF SELENIUM IN BIOLOGICAL MATERIAL

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SUMMARY.

A method for the determination of selenium in trace quantities in a variety of animal and plant tissues is described. The method makes use of the fluorescence of the selenium-diamino benzidine complex in hydrocarbon solvents. Determination of as little as 0·02 μg selenium is possible and the method has been routinely used for estimations in the range 0·1-2·0 μg selenium.

INTRODUCTION.

Until recently interest in the physiological action of selenium has centred on its toxicity. The suggestion that it is essential for some animals, under defined conditions, has revived interest in the biochemistry of the element (Schwarz, 1958). Prevention of muscular dystrophy ('white muscle' disease) in sheep and certain types of hogget ill-thrift by selenium dosage is further confirmation of the metabolic significance of the element (Hartley, 1959). However, knowledge of the metabolism and normal levels of selenium has been severely handicapped by lack of a sufficiently sensitive method of determination.

Classical methods of estimation usually require a wet ashing with sulphuric-nitric acid mixtures, isolation of the selenium from the digest by distillation as the volatile tetrabromide, and determination of the element either gravimetrically, or by means of the oxidising properties of selenious acid. Such methods are generally not applicable to analysis of microgram quantities. The discovery of 3,3′-diamino benzidine (DAB) as a specific complexing agent for selenium (Hoste and Gillis, 1955) and photometric determination of the element by means of formation of the selenium-DAB complex [5,5′-di(2,1,3-benzoselenadiazole)] (Cheng, 1956) were notable advances in methodology. However, the trace amounts of selenium present in most natural materials coupled with a relatively high light absorption by the blank necessitates fairly large samples for analysis, with attendant disadvantages. The requirement is for a method allowing rapid, concurrent analyses utilising samples of the order of 1-5 gm. dry weight.

A study of wet and dry ashing procedures for trace element analysis using

isotopes of the elements showed digestion mixtures incorporating perchloric acid to be the most suitable for selenium determination (Gorsuch, 1959). An earlier paper likewise indicated probable losses of the element by H_2SO_4/HNO_3 digestion of the samples (Fogg and Wilkinson, 1956). In the present work the advantages of wet digestion utilising perchloric acid mixtures were confirmed. Such mixtures have the added advantage of speed in digestion and, because of the high solubility of most perchlorates, of preventing the appearance of troublesome precipitates of salts. Attempts to isolate microgram quantities of selenium from the digest by distillation proved unsuccessful. Coprecipitation with arsenic as used by Luke (1959) gave quantitative recovery of trace amounts of the element.

In an effort to find a more sensitive method of estimation of selenium the fluorescence of the Se-DAB complex was investigated. In aqueous solution or in polar solvents such as n-butanol, isobutanol, isoamyl alcohol and amyl acetate no useful fluorescence was shown. However, in hydrocarbon solvents (toluene and xylene) the complex showed a rather unusual brown fluorescence, although not of high intensity, when illuminated by a Woods lamp. Under similar circumstances diamino benzidine showed only a weak blue fluorescence. Fluorimetric determination of selenium by means of the Se-DAB complex proved at least twenty times more sensitive than photometric estimation.

The method used for the isolation of selenium and formation of complex is essentially a modification of the procedure described by Luke (1959). The selenium may be estimated photometrically by measurement of absorption at 420 mμ in the range 2-50 μg. Se or fluorimetrically at levels below 4 μg. Se.

Materials and Method.

Reagents:

1. Standard selenium solution. A solution of selenious acid equivalent to 1 mg. selenium per ml. This stock solution is standardised gravimetrically and diluted to give a standard containing 4 µg. selenium per ml.
2. Digesting mixture (I). A mixture of 1 part 70 p.c. perchloric acid and 3 parts nitric acid, sp. gr. 1·42, for wet ashing of sample.
3. Arsenic solution. Arsenious oxide, 0·25 gm., plus 10 pellets of NaOH is dissolved in 10 ml. of water with warming and diluted to 200 ml.
4. Reducing solution. A solution of 2 parts 50 p.c. hypophosphorous acid, 1 part concentrated hydrochloric acid and 3 parts water.
5. Digesting mixture (II). A mixture of 1 part 70 p.c. perchloric acid and 9 parts nitric acid, sp. gr. 1·42, for dissolution of precipitate and ashing of filter paper.
6. m-Cresol purple indicator. 0·1 p.c. solution in water.
7. Formic acid. 2·5 M.
8. Ethylene-diamine-tetraacetic acid. 0·1 M.
9. Diamino-benzidine-tetrahydrochloride. 0·3 p.c. solution prepared fresh daily.

Digestion.

The sample (1-10 gm. wet weight for most animal tissues) is weighed into a 500 ml. Erlenmeyer flask, 20 ml. digesting mixture (I) added and heated gently on a sand bath or hot plate. On further heating copious brown fumes of nitrogen oxides are evolved and the nitric acid is distilled off. As the last of the nitric acid boils off the reaction becomes more vigorous and should be controlled by removal from the hot plate if necessary. Heating is continued until white fumes of perchloric acid occupy the flask and the digest becomes clear and colourless.

Larger samples may be successfully ashed by increasing the quantity of nitric acid added. It is important that there be excess nitric acid present and that when the digest enters the perchloric acid phase the bulk of the organic matter be oxidised. Frothing in the initial stages of the digestion may be controlled by the addition of distilled water. Should salts start to precipitate during the digestion, the digest is removed from the hot plate, cooled and a further 5-10 ml. perchloric acid added to prevent the contents solidifying during heating.

Isolation of selenium.

If after cooling the digest the salt deposit is considerable, the precipitate is removed by filtering through a pad of glass wool, and washing the flask and pad with 5N HCl. To the filtrate and washings, or to the perchloric acid residue of the digest are added 1 ml. arsenic solution and 30 ml. reducing solution. The solution is heated to give five minutes' gentle boiling, bringing about coprecipitation of elemental arsenic and selenium. It is to be realised that the combination of a strong oxidising agent such as hot perchloric acid and an active reducing substance such as hypophosphorous acid is an extremely dangerous one. Explosion could occur if the concentration of the perchloric acid exceeded approximately 70 p.c. To obviate this possibility the following precautions are in force in this laboratory: addition of reducing agent in a dilute form, heating for a clearly determined time interval and use of an 8-in. water jacketed condenser seated loosely in the neck of the flask during heating. The digestion and precipitation procedures are carried out in a protected fume cupboard.

The precipitate is isolated by filtering through a 9 cm. Whatman No. 42 filter paper, the flask and precipitate being thoroughly washed with small quantities of 5N HCl. Solution of the precipitate and removal of the paper are achieved by a second digestion. The paper plus precipitate is transferred to an 8-in. x 1-in. micro-Kjeldahl flask and 15 ml. digesting mixture (II) added. The digestion is most conveniently carried out on a micro-Kjeldahl digestion rack, with the tubes in a vertical position and using a manifold attached to the water pump for fume extraction. Digestion is again continued until the white fumes of perchloric acid fill the flask and the digest is clear and colourless.

Estimation.

The digest is quantitatively transferred with small washings of distilled water to a 6-in. x 1-in. glass stoppered tube marked at 15 ml. The tube contents are made up to this mark with water, 1 drop of m-cresol purple indicator added, and adjusted just to the yellow of the indicator by the cautious addition of concentrated ammonia. 2 ml. formic acid, 1 ml. EDTA and 1 ml. DAB are then added and the solution let stand for 45 minutes for complex development. On completion of this the solution is adjusted to pH 5-6 by the drop-wise addition of concentrated ammonia (just to the first purple shade of the indicator). Six ml. of toluene are added and the Se-DAB complex extracted by vigorous shaking for one minute. The toluene extract is transferred to a clean test tube and dried by the addition of 1 gm. anhydrous sodium sulphate and read.

Blanks and 'high' standards are carried through the same procedure.

Fluorimetric readings were carried out by means of a Locarte 13 stage photomultiplier, single-sided fluorimeter. With this instrument the galvanometer is adjusted to zero on the blank and set to give maximum deflection on the 'high' standard.

DISCUSSION.

In the absence of a spectrofluorimeter it was not possible to determine the activation and fluorescence spectra of the Se-DAB complex and of DAB in toluene. For excitation a filter system giving maximum transmission at 450 mμ was used. At this wavelength fluorescence of the complex is considerably reduced but on exciting at wavelengths below 400 mμ the blank fluorescence is so considerable as to cause difficulty in measurement. The fluorescent emission of the complex in toluene has a maximum at approximately 580 mμ and a filter giving maximum transmission at this wavelength was used on the photocell side of the instrument.

Fluorescence of the complex is stable to irradiation and there is no requirement for an external 'high' standard. Blank fluorescence is stable for at least four hours and it is recommended that readings be completed within this time.

The method has been successfully applied to the determination of selenium in a number of animal soft tissues, bone, teeth, wool, milk, urine, yeast, cereals and grasses. Table 1 gives results of a determination on wheat and recoveries of added selenium at different levels.

TABLE 1.

Determination of Se in wheat with recoveries of added Se (in duplicate).

Sample	Se added (μg.)	Se determined (μg.)	Added Se recovered (p.c.)
Wheat (5 gm.)	nil	0·43, 0·47	—
Wheat (10 gm.)	nil	0·79, 0·85	—
Wheat (5 gm.)	1·00	1·39, 1·44	97
Wheat (5 gm.)	0·40	0·82, 0·73	84

Table 2 gives the standard deviation in estimation at two different levels.

TABLE 2.

Error of estimation.

Recoveries of standard Se at two different levels after treatment according to standard procedure. Ten determinations at each level.

Se added (μg.)	1·00	0·40
Mean recovery (μg.)	0·99	0·42
Range of recoveries (μg.)	0·93—1·04	0·35—0·50
Standard deviation	±0·04	±0·05

An analysis requires approximately 9 hours, and 12 determinations can conveniently be carried out concurrently. Strict adherence to procedure and identical treatment of replicates is essential. The sensitivity of the method could be improved by a reduction in blank fluorescence. This could possibly be achieved by chemical means and almost certainly by choice of a suitable monochromatic exciting source as provided by a spectrofluorimeter.

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COXIELLA BURNETI IN KANGAROOS AND KANGAROO TICKS IN WESTERN QUEENSLAND

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SUMMARY.

Complement-fixing antibodies to *Coxiella burneti* were present in 23 p.c. of kangaroos collected in western Queensland, and agglutinins in 15 p.c. Red kangaroos, *Megaleia rufa*, showed a higher incidence of complement-fixing antibodies (33 p.c.) than grey kangaroos, *Macropus major* (12 p.c.). In three areas, the incidence of complement-fixing antibody in *M. rufa* was between 46 and 54 p.c.

C. burneti was isolated in mice from the blood of one *Macropus major*.

Thirteen isolations were made from nearly 3,000 ornate kangaroo ticks, *Amblyomma triguttatum*, collected from kangaroos, goats and sheep. The titre of infectivity of infected ticks for mice was usually in the range 10^{-6} to 10^{-9}.

Infection of kangaroos and *A. triguttatum* was found over a considerable area, between 24 and 28° S. and 144 and 149° E., in western Queensland.

It thus seems probable that a natural kangaroo-tick cycle exists. The three-host life cycle and wide host range of this tick would allow it to spread infection to domestic animals as well as kangaroos. Infestation of sheep would bring infected ticks into contact also with workers in the sheep-shearing industry, and was probably responsible for recent outbreaks of Q fever among them.

INTRODUCTION.

The occurrence of Q fever in men closely associated with shearing in western Queensland was recently reported (Derrick, Pope and Smith, 1959). There seemed to be a high probability that infection was due to the presence of *Coxiella burneti* in the sheeps' wool at the time of shearing, but no decisive information was obtained as to how the wool became contaminated. Ornate kangaroo ticks, *Amblyomma triguttatum*, were present on the sheep at the shearing at which the largest number of cases occurred. This observation led to the suspicion that the ticks may have been carrying rickettsiae, and directed attention to kangaroos as possible reservoirs. Complement-fixing antibodies to *C. burneti* were present in the sera of one of three kangaroos collected in the vicinity of the outbreak.

Dane and Beech (1955) had previously reported the presence of significant levels of complement-fixing antibodies to *C. burneti* in the sera of one of two kangaroos from near Alice Springs, in the Northern Territory, and of one of three kangaroos from Hawker, in South Australia. The sera of 13 young kangaroos from Port Augusta were negative. These authors considered that kangaroos might be important as the permanent reservoir of Q fever in South Australia, as they could find no evidence incriminating domestic animals.

The further studies reported here were carried out to determine the incidence of antibodies to *C. burneti* in kangaroos in western Queensland, and to investigate the possible rôle of *A. triguttatum* in the epidemiology of Q fever.

Materials and Methods.

The animals studied in this work were the red kangaroo, *Megaleia rufa* (Desmarest, 1822) (= *Macropus rufus*), the grey kangaroo, *Macropus major* Shaw, 1800, the swamp wallaby, *Wallabia bicolor* (Desmarest, 1804), the wallaby, *W. dorsalis* (Gray, 1837), the wallaroo, *Osphranter robustus* (Gould, 1841), and the goat, *Capra hircus* L., 1758. The ornate kangaroo tick, *Amblyomma triguttatum* Koch, which was prevalent on kangaroos, wallabies, goats and sheep in certain districts, and the dog tick, *Rhipicephalus sanguineus* (Latrielle), were also investigated.

Two hundred and ninety-six animals were shot. The species and sex were noted, the ratio of male to female marsupials being 3:2.

Blood was collected into a sterile McCartney bottle, either from the severed carotid or jugular vessels, or directly from the heart after quickly opening the thorax. Sera were not separated in the field, the blood samples being despatched by air to the Laboratory of Microbiology and Pathology, Brisbane, where the serological tests for antibodies to *C. burneti* were performed. Sera were tested for complement-fixing (CF) antibodies by the method of Lennette *et al.* (1949) using Lederle antigen (American strain—Nine Mile), and also for agglutinins, using C.S.L. Agglutinating Suspension (Henzerling strain) and equal quantities of two-fold dilutions of serum. Periods up to three days elapsed between collection of blood and removal of the sera. During the first two collection trips (June and October, 1958) blood samples were refrigerated whenever possible before despatch, while during the third period of collection (December, 1958) they were kept chilled in an insulated box containing "Magic Briks" which had been previously frozen.

After removal of the serum, blood clots from 24 of the kangaroos were individually ground, suspended in saline and inoculated intraperitoneally into a group of three or six weaned mice. Three serial intraperitoneal passages, using liver and spleen as inoculum, were then made.

The ticks removed from the animals were despatched to the Queensland Institute of Medical Research within three days. In the laboratory, they were held in the refrigerator at approximately 4° C. until identified and counted, and were then stored in a "Revco" Freezer (—70° C.) until tested. Each batch of ticks from one host was weighed (except in the early part of the work), ground separately with alundum, and suspended in saline. A pool, usually representing 50-100 ticks, was then made up from portions of each batch suspension. The remaining portions of all suspensions were held at —20° C. Penicillin was added to the pool in a concentration of 400 units per ml., and the mixture allowed to stand for 30 minutes at 4° C. before inoculation into guinea-pigs and weaned and suckling mice.

Three serial seven-day intraperitoneal passages were made from three of the six weaned mice inoculated intraperitoneally with the tick suspension, and three serial fourteen-day

intraperitoneal passages were made from the remaining three mice. The inoculum was prepared from liver and spleen. Weaned mice were also inoculated intracerebrally with the tick suspension, and three serial intracerebral seven-day passages, using brain as inoculum, were made. One of the two litters of suckling mice was passaged (liver, spleen and brain, intraperitoneally) at 7 days into another litter. Otherwise, litters were observed for twenty-one days. One of the two intraperitoneally inoculated guinea-pigs was sacrificed at seven days, and a suspension of its liver and spleen inoculated intraperitoneally into two further guinea-pigs. Sera were collected from the three guinea-pigs between five and six weeks after inoculation, and were tested for the presence of CF antibodies to *C. burneti*.

When mice succumbed to the toxicity of the inoculum within 24 hours, the suspension was diluted between five and 25 times, depending on its apparent concentration, and the inoculation was repeated.

If transmissible splenomegaly occurred in the mice, impression smears of spleen were stained with Giemsa and searched for rickettsiae. In confirmation, infective mouse tissues were inoculated into two guinea-pigs whose sera were subsequently tested for CF antibodies. If *C. burneti* was isolated from a pool, the stored samples of each batch of ticks in the pool were then tested individually by inoculation into mice.

The inoculum from an infected batch of ticks was also titrated in mice. A 1/100 suspension was first prepared from the ticks, and from it further tenfold dilutions were made. The diluent was saline containing 400 units of penicillin per ml. Each dilution was inoculated intraperitoneally (0·5 ml.) into a group of three mice, which were examined at seven days. Two further serial seven-day passages were made. A dilution was considered positive when splenomegaly occurred and rickettsiae were demonstrated in spleen smears of the subinoculated mice, and negative when the three passages showed no splenomegaly.

In some instances, batches of ticks were washed in 10 ml. of saline, and the washings were stored at — 20° C. In titrating these washings, tenfold dilutions were made, and inoculations were performed as with the titrations of tick suspensions.

RESULTS.

Serological findings.

The results of the CF and agglutination tests for antibodies to *C. burneti* in the sera of kangaroos, wallabies, and wallaroos, are shown in Table 1. Fifty of the 270 sera (18 p.c.) examined possessed either CF or agglutinating antibodies or both. Twenty-two per cent. of the sera which were effectively tested possessed CF antibodies at a titre of 1/8 or more, while 14 p.c. of sera possessed agglutinins at similar levels. If the results for *M. major* and *M. rufa* are grouped, the incidence of CF antibodies was 23 p.c. and of agglutinins 15 p.c. Further analysis of the results of two tests is set out in Tables 2 and 3, from which it can be seen that 21 of the 50 positive sera possessed both CF and agglutinating antibodies, and that titres ranged up to 256 and 128, respectively. The results of each test also show an overall greater incidence of specific antibody in *M. rufa* than in *M. major*. In three areas, Augathella, Tambo and Blackall, the incidence of CF antibodies in *M. rufa* was 50, 46 and 54 p.c., respectively, while for the first two areas the incidence in *M. major* was only 16 p.c. (Table 4 and Fig. 1). There were no *M. major* in the Blackall series. A similar incidence of

TABLE 1.

Results of complement fixation and agglutination tests on sera of kangaroos, wallabies and wallaroos.

Species	No. of sera tested	No. with anticomplementary or non-specific reaction	Incidence of complement fixing antibody (at titre of 1/8 or more) in sera effectively tested		Incidence of agglutinating antibody (at titre of $\frac{1}{4}$ or more)	
			Proportion	Percentage	Proportion	Percentage
M. rufa	95	20*	25/75 } 33/141	33 } 23	28/95 } 38/251	29 } 15
M. major	156	90	8/66 }	12 }	10/156 }	6 }
W. bicolor	10	4	0/6	0	0/10	0
W. dorsalis	2	2			0/2	0
O. robustus	7	4	0/3	0	0/7	0
Total	270	120	33/150	22	38/270	14

* The one non-specific reaction recorded was in this group.

TABLE 2.

Analysis of results of complement fixation and agglutination tests on the sera of kangaroos, wallabies and wallaroos.

Complement fixation test result	Agglutination test result		Total
	Titre of 1/8 or more	Titre < 1/8	
Titre of 1/8 or more	21	12	33
Titre < 1/8	3	114	117
AC or non-specific	14	106*	120
Total	38	232	270

* The one non-specific reaction recorded was in this group.

TABLE 3.

Distribution of antibody titres in kangaroo sera.

Reciprocal of titre	8	16	32	64	128	256	512	Total
No. of sera with complement fixing antibodies	2	12	5	5	7	2	—	33
No. of sera with agglutinins	6	11	12	6	3	—	—	38

TABLE 4.

Geographical distribution of kangaroos with antibodies to C. burneti.

District	*M. rufa*		*M. major*	
	CF antibodies	agglutinins	CF antibodies	agglutinins
Inglewood-Talwood	0/2	0/2	0/0	0/9
St. George	—	—	0/4	0/12
Surat	—	—	0/0	2/2
Roma	0/1	0/2	2/10	0/25
Muckadilla	3/7	4/9	3/27	2/44
Mitchell-Morven	1/4	1/5	0/1	0/3
Charleville	0/1	1/2	0/1	0/7
Augathella	6/12	5/13	1/6	0/22
Tambo	7/15	8/20	2/12	6/25
Blackall	7/13	7/14	—	—
Quilpie	0/8	2/12	0/1	0/1
Cunnamulla	1/12	0/16	0/4	0/6
Total	25/75	28/95	8/66	10/156

— = No sera collected from this species.

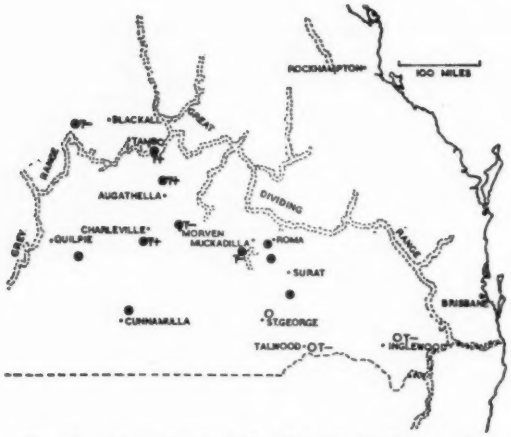

Fig. 1. *Map showing the districts studied, and the distribution of kangaroos with antibodies and of infected ticks.*
● = kangaroos with CF or agglutinating antibodies.
○ = kangaroos without CF or agglutinating antibodies.
T+ = ticks infected with *C. burneti.*
T– = ticks not infected with *C. burneti.*

antibodies was shown in each type of test for the areas studied. The incidence of CF antibodies was similar in males and females. No antibodies were detected in the sera of 12 wallabies, 10 *W. bicolor* and two *W. dorsalis,* and of seven wallaroos, *O. robustus,* effectively tested (Table 1).

Complement fixation tests were also carried out on the sera of 26 wild goats. Twenty-one were captured at Muckadilla (3-6.10.58), two at Augathella (8.12.58), and three at Tambo (15.12.58). The results were negative.

The incidence of anticomplementary reaction in the marsupial sera was high (Table 1). Approximately 44 p.c. were anticomplementary, and the proportion was much higher in grey kangaroos, wallabies and wallaroos (100/175) than in red kangaroos (20/95). On our present information, it is not possible to decide whether kangaroo and wallaby sera would show such a large proportion of anticomplementary reactions even if collected under ideal circumstances, or whether they simply deteriorate rapidly, with production of anticomplementary factors. Species variation in the marsupials is shown by the marked difference in incidence of anticomplementary reaction between sera from red and grey kangaroos. In contrast, the sera of the wild goats were entirely satisfactory for use in the CF test, although they were collected and handled under the same field conditions as those from the marsupials.

Isolation of C. burneti *from kangaroo.*

C. burneti was isolated from one of the 24 samples of kangaroo blood tested, and the titre of the blood was 10^{-2} (Table 6). The infected kangaroo was a male *M. major* captured in the Augathella district. The serum was anticomplementary, and the agglutination test was negative. This isolation of *C. burneti* confirms that infections were actually present in the kangaroos.

Isolation of C. burneti *from ticks.*

A total of 2,965 ticks was investigated. Thirteen strains of *C. burneti* were isolated by mouse and guinea-pig inoculation from 2,197 *A. triguttatum* (adults except for six nymphs), collected in December, 1958 (Table 5). Of these strains, nine were obtained from ticks from kangaroos, two from ticks from goats, and two from ticks from sheep. The results for the Augathella district show that the incidence of infection was higher in ticks from *M. rufa* than in those from *M. major* (Table 5). Complete correlation was found between the results of mouse and guinea-pig inoculation; all pools of ticks which were infec-

TABLE 5.

Isolation of C. burneti *from* A. triguttatum *collected in December,* 1958.

Host	Locality	No. of batches of ticks tested	No. of ticks	No. of batches with *C. burneti*
M. major	Inglewood-Talwood	7	31	0
M. rufa		2	2	0
W. dorsalis		2	11	0
M. major	Muckadilla	14	113	0
M. rufa		2	4	0
M. major	Mitchell-Morven	3	5	0
M. rufa		3	13	0
M. major	Augathella	19	384	1
M. rufa		10	355	6
Goat		2	89	2
Sheep		1	68	1
M. major	Tambo	18	171	1*
M. rufa		18	139	
Goat		1	2	0
Sheep		1	621	1
M. rufa	Blackall	14	154	0
M. major	Charleville	1	26	1
Man	Various	4	9	0
Total		122	2197	13

* An isolation was made in both mice and guinea-pigs from the pool of ticks from 4 *M. rufa* and 4 *M. major*. However, the concentration of rickettsiae was apparently low, because when the 8 batches were later tested individually, all were negative.

TABLE 6.

Concentration of C. burneti *in* A. triguttatum, *and in kangaroo blood, and the results of serological tests on the hosts' sera.*

Source	Titre[1]	Reciprocal complement fixing antibody titre	Reciprocal agglutinin titre
Ticks from Kangaroo 137, *M. rufa* ♂	10^{-7}	Anticomplementary	64
Ticks from Kangaroo 138, *M. major* ♂	$>10^{-8}$	Anticomplementary	0
Ticks from Kangaroo 140, *M. rufa* ♂	10^{-6}	16	0
Ticks from Kangaroo 141, *M. rufa* ♂	$<10^{-2}$	0	0
Ticks from Kangaroo 142, *M. rufa* ♂	$>10^{-8}$	128	128
Ticks from Kangaroo 145, *M. major* ♂	10^{-3}	Anticomplementary	0
Ticks from Kangaroo 198, *M. rufa* ♂	10^{-7}	0	0
Ticks from Kangaroo 200, *M. rufa* ♂	10^{-7}	128	32
Ticks from Goat 46, ♂	10^{-6}	0	N.T.
Ticks from Goat 47, ♂	10^{-7}	0	N.T.
Ticks from sheep (Augathella)	10^{-9}	N.T.	N.T.
Ticks from sheep (Tambo)	10^{-8}	N.T.	N.T.
Washings of ticks from sheep (Tambo)	0		
Blood of Kangaroo 271, *M. major* ♂	10^{-2}	Anticomplementary	0

[1] = Expressed as the maximum dilution of which 1·5 ml. was infective for mice.
N.T. = Not tested.

tive for mice also produced CF antibodies in guinea-pigs, as well as a febrile reaction of somewhat variable duration within ten days of inoculation. An irregular febrile response was occasionally produced by inocula from ticks which later proved uninfective. The results of the CF and agglutination tests on the sera of animals from which infected ticks were collected are also given in Table 6.

The results of the titration in mice of 12 infected batches of *A. triguttatum* are listed in Table 6, and show that the concentration of rickettsiae in the infected ticks generally reached high levels. Ten out of 12 of the titres were in the range 10^{-6} to 10^{-9}, while one was 10^{-3} and another $< 10^{-2}$. High titres were found in ticks from each host, kangaroo, goat and sheep. The kangaroos and goats from which infected ticks were taken were all males, but it is not thought that this is of any epidemiological significance, as there were four times as many males as females in the kangaroos studied in that area.

Comparison of the incidence of infected batches of ticks from kangaroos from different localities (Table 5) suggests that, at the time of study, *C. burneti* was most widespread in *A. triguttatum* at Augathella.

No definite investigation was made of the number of infected ticks in the infected batches. However, some information on this point was obtained from the inoculation of the 621 ticks from sheep at Tambo. These ticks were pooled, and divided randomly into six groups (each of approximately 100 ticks) for inoculation. *C. burneti* was isolated from five of the six groups, and the fact that one was negative suggests that the proportion of infected ticks in each group was probably low.

One of these infected groups of ticks collected from Tambo sheep was tested for external contamination by the method used by Stoker and Marmion (1955). Both the ticks and their washings were titrated, and, whereas the ticks had a titre of 10^{-8}, the washings proved uninfective (Table 6).

No isolations were made from *A. triguttatum* of the first two collections, which included 45 nymphs collected from four kangaroos and three goats captured in the Roma and Muckadilla areas in June, 1958, 370 nymphs collected from 25 kangaroos, five wallabies and one goat at Muckadilla in October, 1958, 125 nymphs collected from sheep south of Surat in October, 1958, and 110 adults collected between December, 1958, and February, 1959, from various parts of Queensland through the courtesy of Mr. C. R. Mulhearn, Director of Veterinary Services. In addition, 118 *R. sanguineus* (nymphs and adults) taken from dogs at Tambo in December, 1958, were also negative.

No signs of the presence of any infectious agents were observed in the intracerebrally inoculated weaned mice or the intraperitoneally inoculated suckling mice. It appears that infant mice are not very susceptible to *C. burneti*.

DISCUSSION.

It is evident from this work that kangaroos possessing antibody to *C. burneti* are distributed over a considerable area of western Queensland. Antibodies were present in the sera of kangaroos from 11 of the 14 districts studied (Fig. 1), including the Surat and Blackall districts (almost 400 miles apart), seven areas between them, and the Cunnamulla and Quilpie districts over 100 miles to the south-west. It must be remembered that some migration of kangaroos occurs, and may influence the geographical distribution of those with antibodies to *C. burneti*. Infected *A. triguttatum* were found in three districts. The Tambo and Augathella districts are 50 miles apart, and the third, near Charleville, is almost 100 miles further south. Thus, infected kangaroo ticks also have a wide distribution in the area.

The natural infection of *A. triguttatum* with *C. burneti* is a significant new finding, because it is an abundant, three-host tick, with a wide host range, and wide geographical distribution. As discussed later, it is probable that ticks may infect kangaroos. Maintenance of infection in both kangaroos and ticks, from one tick generation to the next, would be assured if transovarial transmission occurred. A further possible means to accomplish this would exist, if instar to instar passage occurred, as is usual in ticks (Stoker and Marmion, 1955), and the rickettsaemia produced in kangaroos by the last of the infected adults were to infect the first larvae of the succeeding generation. Observations suggest that there is sufficient overlap of the annual tick generations for this process to occur, and it would be reinforced if instar → kangaroo → instar transmission also occurred. Once *C. burneti* became established in some of the larvae, infection

of further kangaroos, and later instars of the ticks would follow automatically, unless the immunity rate in the kangaroo population was high.

As also noted by Stoker and Marmion (1955), the possibility must be considered that isolation of rickettsiae from the ticks may have been a result of contamination, perhaps from infected excreta of the host, or from some other source. In this work, the results obtained in titrations of various batches, and the fact that the washings of one batch were negative, are strong evidence against this possibility. The titres of 10^{-6} and higher recorded could scarcely have resulted from contamination.

There is a second kangaroo tick, *Ornithodoros gurneyi* Warburton, in inland Queensland (Roberts, 1952, p. 210), but it was not encountered in this study. Smith (1942) found that a small number of *O. gurneyi*, which were fed on infected guinea-pigs, failed to transmit the infection by feeding on fresh guinea-pigs, although *C. burneti* was later recovered from the ticks by animal inoculation. These two quite different species need to be distinguished in epidemiological studies, owing to the confusion that can arise from their having the same common name.

The area investigated lies in the sheep grazing region of Queensland. To the west lies the great expanse of channel country, where cattle grazing is carried on. To the east of the Great Dividing Range lies another large area also given over predominantly to cattle. There are also smaller numbers of dairy and beef cattle and horses in the sheep belt. Kangaroos are by far the most numerous of the wild mammals. Goats were originally domesticated in country towns, but some have run wild, and multiplied considerably. Herds of several hundred are occasionally rounded up and destroyed. There is thus a considerable population of domestic and wild animals, which are known to be susceptible to Q fever, and are potential reservoirs.

Of these possible hosts in the area studied, cattle, sheep and kangaroos have shown evidence of infection, but none has been obtained in goats. Some information has been gained about the respective rôles of these reservoirs and of *A. triguttatum* in the epidemiological picture. It is known that cattle are efficient reservoirs, and that ticks may not be essential for *C. burneti* to be enzootic. Transmission is usually considered to be by way of infected placentas, with contamination of the environment, and subsequent transfer of infection by inhalation of dust (Derrick, 1953). Similar mechanisms are generally considered to operate in the maintenance of *C. burneti* in sheep in California (Derrick, 1959). It is possible, however, that some of the infections of sheep and cattle in western Queensland result from tick bite, as *A. triguttatum* will feed on both (Roberts, 1952, p. 220). In the case of kangaroos, the findings reported here strongly suggest that ticks are largely responsible for enzootic infection, and that a kangaroo-tick cycle occurs, as forecast by Dane and Beech (1955). Nevertheless, demonstration of transmission of *C. burneti* by *A. triguttatum* would

be necessary to prove that a cycle exists, and the possibility that kangaroos may become infected from airborne rickettsiae originating from cattle placentas, or from other sources, cannot as yet be excluded.

It seems probable, then, that tick transmission occurs in kangaroos and airborne transmission in sheep and cattle, and that each may be supplemented by overflow from the other. Widespread dissemination of ticks infected with *C. burneti* is possible, because of the ease with which kangaroos range the countryside. The entry of infected kangaroos or ticks into an area containing numerous susceptible hosts could be followed by a sharp increase in the local incidence of infection in both mammals and ticks. The fact already mentioned that *A. triguttatum* requires three hosts to complete its life cycle would increase its effectiveness in spreading infection.

Infection in kangaroo shooters has not been observed, and it seems likely that the significant link between man and the kangaroo-tick cycle is more indirect. The available evidence indicates that shearers become infected with rickettsiae from infected ticks infesting the sheep, rather than with rickettsiae excreted by the sheep during active infection. Part of the reason for the efficacy of this link may lie in the nature of shearing (Derrick *et al.*, 1959), during which infected ticks may be readily cut and crushed, thereby contaminating dust and wool fibres, which are continually being dispersed into the air by the constant activity. Percutaneous infection through scratches from burrs in the wool may also occur.

Thus, the present work adds to current concepts of the epidemiology of Q fever, as it reveals a new significance of ticks of wide host range, in that infected ticks may be taken into contact with man by a host whose rôle may be merely that of mechanical carrier.

Acknowledgments. Thanks are due to Mr. R. Domrow, of this Institute, and Mr. P. J. O'Sullivan, of the Animal Research Institute, Yeerongpilly, for the identification of ticks, to Mr. Don Vernon, of the Queensland Museum, for assistance in the identification of kangaroos, and to Mr. H. E. Brown, of the Laboratory of Microbiology and Pathology, who helped in the agglutination tests. Mr. L. Daniels and Mr. A. J. Carroll contributed greatly to the field work, and the investigation was helped by the many cooperative graziers who allowed collection of material on their properties. Criticism of the manuscript by Dr. E. H. Derrick and Dr. I. M. Mackerras was appreciated.

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PROPERTIES OF THE OLFACTORY EFFERENT SYSTEM

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SUMMARY.

Appropriately timed afferent stimulation of one olfactory bulb can depress afferent induced activity in the opposite bulb, the depression being mediated by the true commissural component of the anterior commissure which is part of the olfactory efferent system. Bulbar retrograde responses evoked by lateral olfactory tract stimulation are depressed by continuous high frequency stimulation of the anterior commissure, and this is considered to be further evidence for a depressive action of the efferent system in the olfactory bulb. Stimulation of the efferent system evokes monophasic negative waves in the bulb which, on continuous stimulation, summate to give a sustained negativity. The appearance of this negative shift is correlated with the onset of the depressive influence. Lateral olfactory tract stimulation evokes polyphasic, dominantly positive, waves in the bulb which do not summate but rapidly fatigue on repetitive stimulation at high frequencies.

INTRODUCTION.

There is evidence that the primary olfactory relay is depressed when the olfactory efferent system of Cajal (Cajal, 1911) is activated by electrical stimulation of the contralateral bulb (Walsh, 1959), the anterior commissure, or the basal rhinencephalon (Kerr and Hagbarth, 1955). The present investigation is concerned with a further examination of the efferent system, some of its properties having been described previously (Walsh, 1959; Kerr and Hagbarth, 1955).

METHODS.

Adult cats were maintained under moderate sodium pentobarbital anaesthesia. The olfactory bulbs were approached from above through the frontal sinus. Sufficient frontal cortex was exposed to allow stimulation of the anterior commissure and lateral olfactory tracts by stereotaxic orientation of bipolar concentric insulated needles. In most experiments electrical activity was recorded from just below the mitral cell layer of the olfactory bulb using a stainless steel needle with a 350μ shaft and a tip diameter of 25-50μ. Some observations were made using finer etched needles with 5-10μ tips. A differential d.c. preamplifier and oscilloscope system were used for recording and display of the evoked potentials. The indifferent electrode was placed for minimal stimulus artefact. A conventional EEG machine and penwriter were used to record olfactory induced waves from the bulb. Stimuli were obtained from Grass stimulators.

In some experiments curarised preparations were used in the recording of olfactory induced waves and evoked responses. After operative preparation under ether, all exposure

margins and pressure points were infiltrated with procaine. The animal was immobilised by intravenous injection of *d*-tubocurarine and maintained on artificial respiration. The evoked potentials to be described were first seen in such curarised preparations, but these often showed a high level of spontaneous activity in the olfactory bulb which interfered with the interpretation and recording of the evoked responses. Barbiturate anaesthesia was more satisfactory in that it did not significantly alter the character of the evoked responses yet depressed the spontaneous activity.

RESULTS.

Anatomical.

Fig. 1, based on Cajal (*loc. cit.*) and Allison (1953), summarises the relevant features of the connections found in the olfactory bulb. The principal dendrites of mitral cells (M_1, M_2) and tufted cells (T_1, T_2) synapse with incoming olfactory nerve fibres in the glomeruli of the external layers of the bulbar cortex. The axons of these cells constitute the olfactory tracts. Mitral axons form the lateral olfactory tract and terminate in the ipsilaterial prepyriform cortex. Tufted cell axons form the anterior commissure which partly terminates bilaterally in the rhinencephalon, while a significant contribution completely crosses to terminate in the opposite bulb. Recurrent collaterals (R_LR_A) on the axons of mitral and tufted cells are directed towards the accessory dendrites of adjacent cells within the bulbar cortex. Deep to the mitral layer there are very large numbers of small granule cells (G) which terminate also in the region of the accessory dendrites of mitral and tufted cells. In the anterior commissure (AC) there are thick efferent fibres from the rhinencephalon, and thin efferent fibres from the contralateral bulb which synapse with these granule cells. In this way mitral and tufted cells of the primary olfactory relay in each bulb are under the influence of the rhinencephalon and the contralateral bulb.

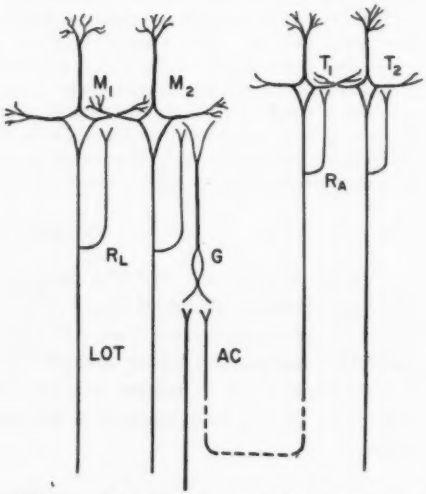

Fig. 1. *Diagram of principal cell types and their connections in the olfactory bulb.* Mitral cells are shown as though in one bulb and tufted cells in the opposite bulb. The broken line indicates the anterior commissure joining the two bulbs. The entire system is, in fact, duplicated in the two bulbs. Abbreviations in this and subsequent figures: AC—anterior commissure, consisting of two efferent parts—a true commissure joining the bulbs, and the efferent system proper; G—granule cell; LOT—lateral olfactory tract; M_1, M_2—mitral cells; R_A—recurrent collateral of anterior commissure; R_L—recurrent collateral of lateral olfactory tract; T_1, T_2—tufted cells.

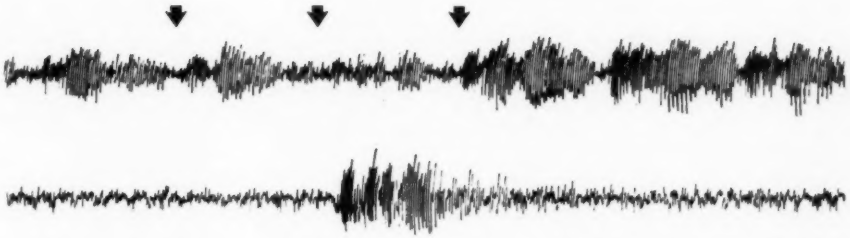

Fig. 2. *Strong olfactory stimulation of one bulb depresses afferent induced activity in the contralateral bulb. Upper record*: Afferent induced activity elicited every two seconds in the left bulb by a brief puff of air in the left nostril. *Lower record*: Right bulbar response to a strong olfactory stimulus in the right nostril, timed to occur just after the third stimulus of the left side. Curarised preparation. The arrows, two seconds apart, indicate the moment of stimulation in the left nostril.

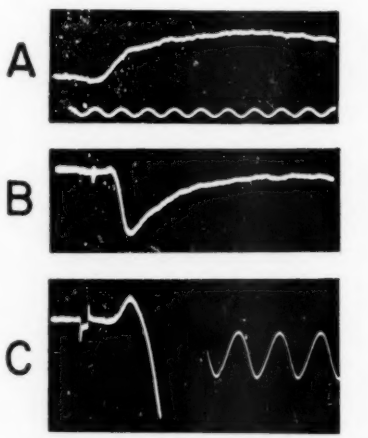

Fig. 3. *Responses evoked in the olfactory bulb.* Stimulation of (A) the anterior commissure, the response outlasts the sweep duration, and of (B) the lateral olfactory tract. Time marker in (A), 200/sec., refers to (A) and (B). (C) More intense stimulation of the lateral tract, only the initial negativity and descending phase of the positivity recorded; time marker 200/sec., 1mV.

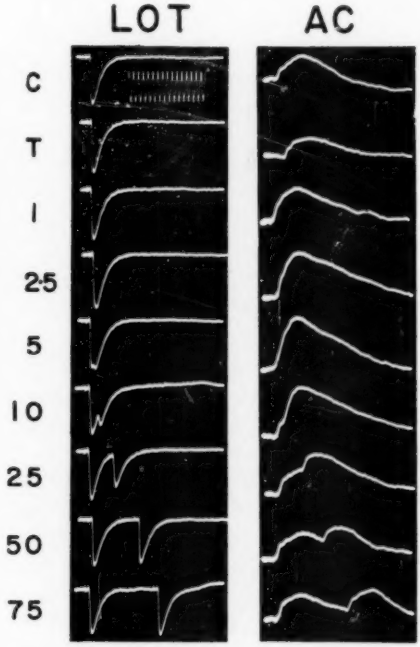

Fig. 4. *Paired shock stimulation of anterior commissure, AC, and lateral olfactory tract, LOT.* The conditioning response, C, is followed by a test response, T, at intervals in msecs. indicated to the left of the records. Time 200 per second, 1 mV.

Bulbar afferent interaction.

Through the commissural interconnection between the two bulbs, afferent activity in one bulb should influence that in the other. Direct stimulation of the commissure depresses afferent induced bulbar activity through the efferent system (Kerr and Hagbarth, 1955), so that afferent activation of the bulbar interconnection would be expected to do the same. This is illustrated in Fig. 2, where, using a curarised preparation, the synchronised activity regularly induced in the left bulb by weak olfactory stimulation was depressed when strong afferent stimulation was applied to the opposite bulb. Afferent induced waves appeared only on the side being stimulated. The stimulus in the right nostril occurred after that in the left so that the effect was not due to impeded air flow in the left airway during high pressure in the nasopharynx owing to the strong stimulation of the right side.

Bulbar responses to commissure stimulation.

A single stimulus to the anterior commissure evoked a long-lasting monophasic negative-going wave with a latency of 4-5 msecs. (Figs. 3, 4, 7 AC). The negativity was recorded throughout the depths of the bulb, but attenuated in the superficial layer, particularly when the finer pick-up needle was used. Occasionally the double-peaked wave reported by Walsh (*loc. cit.*) was seen but the conditions leading to its development remain to be determined. The monophasic negativity was evoked in the bulb by stimulation of any part of the commissure, i.e. lateral limbs, anterior limbs, or at the midline in the commissure proper. Generally the ipsilateral anterior or lateral limb has been used, and in the latter case only the thick fibred commissural efferent component is present.

Paired shocks (Fig. 4) showed that the responses evoked in the bulb by commissure stimulation had a short refractory period, less than 2 msecs., probably the refractory period of the efferent fibres themselves. Amplitude summation occurred when the shock separation was greater than 2 msecs. The summation of test and conditioning responses was apparent so long as the test shock came during the time of the rising phase of the conditioning response (shock separations less than 25 msecs.). Two peaks appeared on the response when greater separations were used, and two separate responses were evoked when the separation exceeded the duration of the conditioning response, 50 msecs. There was no evidence of after-depression or augmentation.

Sustained bulbar potentials evoked by repetitive commissure stimulation.

Summation of repetitive responses occurred so that a sustained negativity was recorded from the bulb. Fig. 5 shows the sustained bulbar potentials developed during commissure stimulation, using a 250 msec. pulse-train at frequencies from 10-100/sec. At frequencies above 10/sec., when the successive responses were evoked during the life-time of preceding responses, the early responses

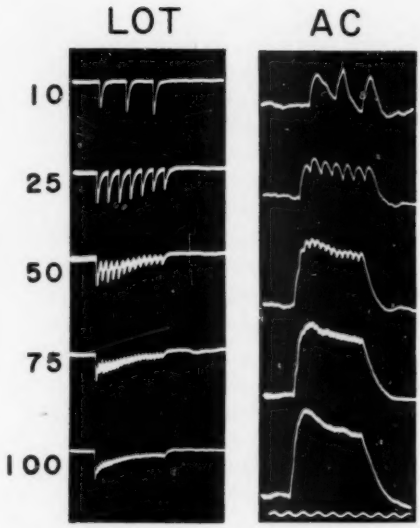

Fig. 5. *Stimulation of the lateral olfactory tract and anterior commissure for a period of 250 msecs. at the frequencies indicated.* Recorded with a slow sweep. Time 20 per second.

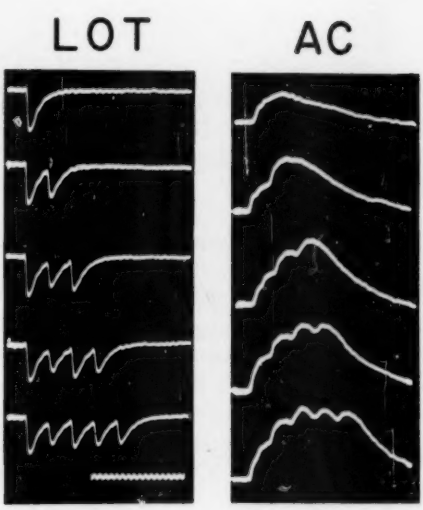

Fig. 6. *Stimulation of the lateral olfactory tract and anterior commissure with a variable-width pulse-train. Frequency*: 50 stimuli per second; one to five stimuli applied to the paths in the records from above downwards. Time 200 per second.

in the train partially summated; the resultant negative shift was sustained by the later responses to the stimulus train. With prolonged stimulation the negativity declined at first rapidly then more slowly, so that there was still an appreciable negative shift present after stimulation for as long as three seconds.

The amplitude summation of the earlier responses in the train was not complete and each successive response added progressively less potential. The later responses merely prolonged the negativity. This was best seen by using a faster sweep and a variable-width stimulus train as in Fig. 6 where

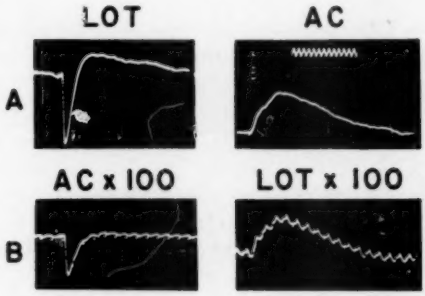

Fig. 7. *Depressive influence of the olfactory efferent system on retrograde lateral tract stimulation.* LOT, single bulbar response (left upper record A) to lateral tract stimulation depressed by continuous stimulation of the anterior commissure at 100 per second (left lower record B). AC, single bulbar response (right upper record A) to commissure stimulation not influenced by lateral tract stimulation at 100 per second (right lower record B). Time 200 per second.

stimuli at the rate of 50/sec. were used and the train gated so that one, two, three, four, or five stimuli were delivered to the commissure. At high frequencies more responses summated so that the negativity was correspondingly greater. Walsh (*loc. cit.*) has reported similar behaviour with the potentials evoked by stimulation of the opposite bulb, the summation being maximal at 30/sec. in his experiments with the rabbit.

Responses evoked by lateral olfactory tract stimulation.

Retrograde stimulation of the lateral olfactory tract evokes high amplitude polyphasic responses in the ipsilateral olfactory bulb (Kerr and Hagbarth, 1955). In Fig. 1 if the axon of mitral cell M_1 were stimulated then M_1 would be antidromically activated while M_2 would be orthodromically activated by way of the recurrent collateral R_L on the axon of M_1. In effect two populations of cells represented by M_1 and M_2 would be so activated, and the retrograde potentials would be due to mixed antidromic and normodromic effects. The bulbar response evoked by a single stimulus to the lateral tract was polyphasic with a latency of 2·5-3 msecs. (Figs. 3, B and C; Figs. 4, 7). There was a small initial negativity which was more apparent at faster sweep speeds with more intense stimulation (Fig. 3, C). The ensuing large positivity was always present, and at times an after negativity developed (Fig. 7 LOT). These responses were recorded from all parts of the bulb so far explored.

The results of paired shock and repetitive stimulation of the lateral olfactory tract are shown alongside each corresponding experiment on the commissure (Figs. 4, 5, 6). The presence of a prolonged subnormality lasting up to 30 msecs. after the conditioning shock, and the ease of fatigue in the retrograde lateral tract response are in sharp contrast with the behaviour of the commissural system, and make it most unlikely that the negativities evoked by commissure stimulation could be due to retrograde tufted cell activation (through the recurrent collateral system analogous to that of the mitral cells, e.g. collateral R_A of T_1 acting on T_2 in the scheme of Fig. 1). The two collateral systems are anatomically so similar that they would almost certainly give rise to similar potentials in the bulb. However, even with the finer pick-up needles no sign of polyphasic retrograde tufted cell responses has yet been seen external to the mitral layer where they would be expected when the commissure is stimulated; although Walsh has described an external positivity in the rabbit bulb following contralateral bulb stimulation.

Interaction between the effects of anterior commissure and lateral olfactory tract stimulation.

It was found that repetitive stimulation of the anterior commissure depressed a concomitant retrograde lateral tract response if the frequency of commissure stimulation was greater than 50/sec. In Fig. 7 stimulation of the anterior com-

missure at 100/sec. markedly depressed the positivity and late negativity of the lateral tract response. In several experiments the retrograde lateral tract response was still at least 50 p.c. depressed after two seconds of continuous commissure stimulation at 100/sec. indicating a slow rate of fatigue in the efferent system.

By contrast, the response to anterior commissure stimulation was not influenced by lateral tract stimulation at 100/sec. (Fig. 7). Only the initial negativity of the repetitive lateral tract response can be seen in the latter record, and appears as a ripple superimposed on the otherwise unaffected slow negative wave of the commissure response. This effect was to be expected from the ready fatigue of the positive component of the lateral tract responses (Fig. 5).

Single shock interactions of the two systems do not produce any significant influence of one on the other. The commissural depressant effect on retrograde lateral tract responses only becomes apparent with frequencies of commissural stimulation which lead to development of sustained negative d.c. shifts in the bulb, i.e. 50/sec. and higher. In the same way commissural stimulation only depressed afferent induced and resting bulbar activity (Kerr and Hagbarth, 1955) at those frequencies which are now seen to give a sustained negativity in the bulb. The action of commissure stimulation on lateral tract retrograde responses confirms that the olfactory efferent system exerts a depressant or inhibitory action on bulbar mitral elements.

DISCUSSION.

The characteristics of the bulbar responses evoked respectively by anterior commissure or retrograde lateral olfactory tract stimulation indicate that two distinctly different mechanisms are involved in their generation. The retrograde lateral tract responses might be antidromic mitral cell potentials, or synaptic due to the recurrent collateral system; there is evidence that they are synaptic (manuscript in preparation). By far the majority of commissural efferent fibres terminate on granule cells, and the evoked responses in the bulb are no doubt due to these cells. Granule cells are small and virtually lack a true axon (Cajal, 1911) so that they might not have a propagated action potential. Walsh has suggested that the negative waves evoked by commissure stimulation are due to non-propagated granule cell activity. However, in some ways the negative waves resemble dendritic potentials, e.g. they behave like the "graded-responses" (Clare and Bishop, 1955) of cortical apical dendrites, particularly in that they summate with repetition to give a sustained d.c. shift. If this analogy is correct then the bulbar potentials arise in the dendrites of mitral cells under the influence of granule cells by a process similar to that in cortical apical dendrites under the influence of short intracortical fibres (Clare and Bishop, 1955). The potentials giving rise to the summation phenomenon would, in this case, originate in mitral dendrites rather than the granule cells themselves.

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THE HAEMATOLOGY OF THE CENTRAL AUSTRALIAN ABORIGINE[1]

IV. HAEMOGLOBIN, ERYTHROCYTE COUNTS, CONSTANTS, FRAGILITIES AND SEDIMENTATION RATES. EFFECTS OF EUROPEANIZATION

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(*Accepted for publication 4th December, 1959.*)

SUMMARY.

Various haematological observations were made on Central Australian aborigines. One group contained some 30 nomadic males living under primitive conditions for most of the year. The second group consisted of about 20 males and 15 females living under urban conditions, on an almost European diet. The nomadic group's diet had a high iron content. The haemoglobin levels of a control group of Europeans in Central Australia were also measured.

The haemoglobin and haematocrit values of the nomads were higher than those of the Bungalow males. Both sexes at the Bungalow did not differ significantly from European controls. The erythrocyte counts showed no significant difference between the two male groups. The haemoglobin levels of a group of 16 Europeans living at Alice Springs were almost the same as those of the Bungalow males.

The M.C.V's and M.C.H's of the nomads were just greater than those of Europeans, but the Bungalow groups did not differ significantly from either the nomads or Europeans. The M.C.H.C's did not differ significantly between the groups or from European standards.

Erythrocyte osmotic fragility studies showed an increase in haemolysis at ·5N but otherwise were not significantly different from those of Europeans. The aborigines did not differ significantly among themselves.

The erythrocyte sedimentation rates were quite raised in all groups of aborigines when compared with Europeans. However, the nomads' values were less than those of the urban groups.

INTRODUCTION.

The Australian aborigine has been the subject of very few haematological surveys. In the first part of this series of papers were recorded the haemo-

[1] The data upon which this work is based were collected during a University of Adelaide Anthropological Expedition. This was financed by grants from the Wenner-Gren Foundation for Anthropological Research, New York, and the Medical Research Fund of the University of Adelaide.

globins, erythrocyte counts, mean corpuscular haemoglobins, red cell diameters and measure of anisocytosis and non-circularity of a group of Central Australian natives. These were living in a semi-detribalized state on a government settlement at Haast's Bluff (Casley-Smith, 1958a).

The results reported in this present paper were obtained at three places. One was again at Haast's Bluff where the subjects were a nomadic group of Pintubis who spent most of the year living in their old ways, but who had just come into the settlement. Unfortunately, owing to religious difficulties, it was impossible to obtain blood from the women. The nutrition of these natives was excellent. The second group of individuals lived at "The Bungalow", a settlement just outside Alice Springs. The natives provided labour for the town, and were fed on an almost European diet, but the women did eat some native foods. Finally, some normal European controls living at Alice Springs were obtained by the Medical Superintendent of the Alice Springs Hospital. These were mostly healthy, but a few had mild diseases which would not affect the haemoglobin levels.

Materials and Methods.

Blood was collected by applying a tourniquet for not more than 30 seconds. Wintrobe's potassium and ammonium oxalate mixture was used as the anticoagulant. The ages were estimated; those at Haast's Bluff being checked by an experienced anthropologist (Mr. N. B. Tindale).

Haemoglobins were estimated at once by the Sahli method as a check. The final estimations were done using a photo-electric colorimeter measuring oxyhaemoglobin produced by dilute ammonium hydroxide. Some of these estimations were performed in the Institute of Medical and Veterinary Science, Adelaide, by Miss F. H. Gaffney. The instrument was in continuous use, and was frequently checked against controls supplied by the National Standards Laboratory, Sydney; these were standardised by the iron content method. The blood was stored in rubber capped bottles in a refrigerator for up to three weeks. This produced no change in haemoglobin values in a control series — cf. Davis and Pitney (1957) who used no refrigeration and observed a rise of 1 gm.

Erythrocyte counts were performed on the oxalated blood, at times in duplicate as a check. They were always done within four hours of collection. The counting chambers and pipettes were standardised, including those used for the fragility estimations. Pack cell volumes were estimated on a 12-inch diameter centrifuge running at 3,000 r.p.m., which produced a constant volume after 30 minutes. The determinations of red cell fragilities were done using the technique described by Whitby and Hynes (1935). The blood was diluted with varying concentrations of buffered saline, and left for 30 minutes. These solutions were checked and had given constant results at the Institute of Medical and Veterinary Science, Adelaide. They were stored in rubber stoppered bottles in a refrigerator. The blood was oxygenated by gentle agitation in a wide-mouthed bottle for five minutes before dilution. Erythrocyte sedimentation rates for one hour were found by the Wintrobe method, within three hours of collection. Corrections were applied for the haematocrit to a standard 45 p.c. (Whitby and Hynes, 1938) and for temperature to a standard 20° C. (Wartman, 1946). The tubes were first overfilled, then adjusted to the mark (Alexander, 1953).

All results were compared using "t" tests unless the variances were significantly different

when Behrens' tests were used (Fisher and Yates, 1953). Throughout this paper the following abbreviations are used to indicate the significance levels:—

N.S. = not significant — $P > \cdot 05$.
X = just significant — $\cdot 5 > P > \cdot 01$.
XX = quite significant — $\cdot 01 > P > \cdot 001$.
XXX = very significant — $\cdot 001 > P$.

RESULTS.

Haemoglobin (Table 1).

The nomads had a level of 16·7 gm. per 100 ml.: those past puberty had 17·1. The Bungalow natives were all post-pubertal. The males' mean was 16·1 and the females' was 13·8. These are compared with the values of 16·6 and 16·5 for men and women at Haast's Bluff found in part I of this paper. They are also compared with the values for Europeans obtained by Wintrobe (1956), Walsh *et al.* (1953) and myself, both at Alice Springs and at Adelaide. The nomads' values were significantly higher than those of the Europeans including those at Alice Springs (X to XXX) and those of the Bungalow males (X). Neither the nomadic nor the Bungalow male group was significantly different from the semi-detribalized Haast's Bluff group, which has been shown to have higher values than Europeans at Adelaide (XX). But this latter group is not significantly different from Alice Springs Europeans. The Bungalow and all European men were not significantly different. The Bungalow women's mean was not significantly different from that of the European women, but was very significantly (XXX) lower than that of the Haast's Bluff women obtained previously.

Erythrocyte counts (Table 1).

The nomads' mean was 5·4 million per cubic mm., the mean of the post-pubertal ones was 5·5. Bungalow males had a mean of 5·5 and females 4·7. Compared to Wintrobe's values the Bungalow males are just significantly raised (X). The nomadic males and Bungalow females did not differ significantly from Europeans of the same sex. As will be explained later, comparison with the present author's previous figures is not valid owing to different techniques being used. This was suspected previously.

Haematocrit (Table 1).

This mean was 50·0 p.c. for all the nomads and 50·7 p.c. for just the post-pubertal individuals. These were very significantly (XXX) greater than the European means. The Bungalow males' mean was 48·0 p.c. and the females' was 42·1 p.c. These were not significantly different from European standards. The nomads' mean was just significantly (X) greater than that of the Bungalow males.

Mean corpuscular volume (Table 1).

The nomads' mean volume was 92·3μ^3 and 91·5 after puberty. The Bungalow males' and females' means were 88·4 and 89·8 respectively, which were

TABLE 1.

		Haemoglobin			Erythrocyte Count			Haematocrit			M.C.V.			M.C.H.			M.C.H.C.		
	Sex	No.	Mean	S.E.	No.	Mean	S.E.	No.	Mean	S.E.	No.	Mean	S.E.	No.	Mean	S.E.	No.	Mean	S.E.
Nomads	M	25	16·71	·330	23	5·42	·124	25	49·98	·832	23	92·35	1·73	23	31·00	·516	24	33·58	·447
Nomads (adult)	M	21	17·08	·328	20	5·54	·119	22	50·56	·833	20	91·50	1·80	20	30·95	·701	21	33·86	·495
Bungalow	M	19	16·13	·239	18	5·51	·098	20	48·03	·778	17	88·41	1·50	17	29·47	·659	19	33·42	·385
	F	14	13·81	·284	13	4·73	·062	14	42·07	·808	13	89·85	1·55	13	29·08	·645	12	32·92	·357
Haast's Bluff (adult) (Casley-Smith, 1958)	M	37	16·65	·185	39	5·11	·142							38	32·91	1·13			
	F	46	16·48	·197	47	4·92	·104							44	33·06	·762			
Europeans at Alice Springs, 1959	M	16	16·20	·240															
Europeans	M		16·00			5·4			47·0			86·5			29·2			33·7	
(Wintrobe, 1956)	F		14·00			4·8			42·0			87·0			28·8			33·4	
Europeans	M	73	15·92	·130	48	4·89	·079							47	32·88	·60			
(Casley-Smith, 1958)	F	83	14·10	·097	57	4·50	·068							57	31·31	·52			
Europeans	M	1645	15·76	·025	Europeans (Lancaster and Banks, 1957)		M	37	43·8		37	89·1	·477	37	33·2	·181	37	37·4	·230
(Walsh, 1953)	F	1142	13·93	·032			F	63	38·3		63	87·9	·806	63	32·4	·252	63	37·1	·239

The "Europeans" of Walsh 1953, Lancaster and Banks 1957, and Casley-Smith 1958 lived in Australia. Where numbers and standard errors are omitted it is because they are negligible when compared with those of the aborigines.

not significantly different from the nomads' value. The mean of the nomads was significantly greater than the European standard (XX), those of both Bungalow groups were not. No group was significantly different from Lancaster and Banks' (1957) values for Europeans in Australia.

Mean corpuscular haemoglobin (Table 1).

The nomads' mean of 31·0$_{\gamma\gamma}$ (adults 30·9) was very significantly (XXX) greater than Wintrobe's figure, but less (XX) than Lancaster and Banks'. The Bungalow males' mean was 29·5 and the females' mean was 29·1, neither significantly different from the nomads' value. The Bungalow values were also less than Lancaster and Banks' (men, XXX; women, XX), but were not significantly different from Wintrobe's. Again, comparison with the present author's previous figures is not valid.

Mean corpuscular haemoglobin concentration (Table 1).

The nomads' mean was 33·6 p.c.; for the post-pubertal ones it was 33·9 p.c. The means for the Bungalow were 33·4 p.c. for men and 32·9 p.c. for women. These were all very significantly (XXX) less than Lancaster and Banks' figures, but were not significantly different from Wintrobe's. The native groups did not differ significantly among themselves.

TABLE 2.
Erythrocyte fragility

Concentration of saline	Total aborigines			Europeans (Dacie, 1954)	Significance of difference
	No.	Mean p.c. intact	S.E.	Mean p.c. intact	
·6	19	97·68	1·72	100	N.S.
·5	20	86·68	2·94	97	XX
·45	20	73·52	6·01	75	N.S.
·4	20	24·87	4·44	32	N.S.
·3	20	2·37	1·18	0	N.S.

Mean p.c. intact indicates the mean percentage of cells left intact compared with the number in normal saline.
Dacie's S.E's were negligible compared with those of the aborigines.

Erythrocyte fragilities (Table 2).

The number of cells remaining in each saline dilution was compared with the number in normal saline. This was expressed as percentages and the statistics calculated for them. The nomads, Bungalow males and Bungalow females were all compared but the differences between them were not statistically significant. Therefore, all aboriginal values were combined and compared with

results for Europeans recorded by Dacie (1954). These results for Europeans were almost identical with those of other workers (Whitby and Hynes, 1935). It seems that the aborigines' cells start to haemolyse slightly earlier. There was a 13 p.c. haemolysis with ·5N saline, with only 3 p.c. for Europeans (XX). The other dilutions did not give significant differences.

TABLE 3.
Erythrocyte sedimentation rates

	Sex	No.	Mean E.S.R.	S.E.	Mean log. E.S.R.	S.E. of log. E.S.R.
Nomads	M	31	15·13	2·03	1·032	·075
Bungalow	M	19	22·61	2·66	1·282	·062
Bungalow	F	13	20·00	1·91	1·279	·042

Erythrocyte sedimentation rate (Table 3).

The mean erythrocyte sedimentation rate of the nomads was 15·1 mm. per hour, that of the Bungalow males was 22·6 and of the women 20·0. These are all very significantly (XXX) greater than Wintrobe's (1956) value of 5·0 for European men and women. There is no difference between sexes as a correction for the haematocrit was applied. None of the correlation coefficients with age was significantly different from zero. Because the distributions were quite skew, more accurate representations were obtained by taking logarithms of each value and performing the statistical analysis on these. This showed all the three means again to be very significantly (XXX) greater than Wintrobe's value which would be 0·477. The two Bungalow groups had values significantly greater (XX) than those of the nomads. (It should be noted that the 95 p.c. limits of the means should be obtained by multiplying or dividing the antilog of the mean log by the square of the antilog of the standard error: not just by adding or subtracting 2 x S.E.)

DISCUSSION.

Haemoglobin, red count and haematocrit.

The nomadic Pintubi males had values greater than those of European males for both haemoglobin and packed cell values. Those of the Bungalow males are not much different from European values, especially when considering those Europeans living at Alice Springs. The red cell counts were elevated but not significantly so. These figures confirm the high haemoglobin levels found previously for aboriginal males in this area (Casley-Smith, 1958). The mean haemoglobin value of the semi-detribalized Haast's Bluff men in 1956 was between those of the Pintubis and of the Bungalow men, though not significantly

different from either of them. The values found by other investigators in other aborigines have been lower than these results, but it is probable that the other investigations have been carried out in areas of poor nutrition or where hookworm is endemic (Wardlaw, 1935; Walsh, 1957; Davis and Pitney, 1957; Horsfall, 1957a). The natives in the present two series were well nourished. There are a number of possible factors causing the high haemoglobin levels in this area: altitude (2,000 feet), dehydration, a diet rich in iron, and hormonal differences. Dehydration was considered as a possibility, since the bush native would usually be living on a minimum of water. This hypothesis is partly supported by the lower values in the Bungalow group, living under an approximation to European conditions. However, the nomads showed no clinical evidence of dehydration when compared to the Bungalow natives, and the mean blood-pressures, serum sodium and serum magnesiums do not support this view (Casley-Smith, 1958b; Casley-Smith and Schwartz, 1958; Charnock *et al.*, 1959). Whatever the causes involved they seem to elevate the nomads' haemoglobins above the rest. The Bungalow natives and Alice Springs Europeans seem to be about the same.

The diet of the natives living under primitive conditions has a very high iron content – from both plants and animals (Dadswell, 1934; McArthur, 1948; Wilson, 1953). The Bungalow males had almost no native diet. It has been shown by some workers that feeding large amounts of iron will raise the haemoglobin levels – especially of women, but also a little in men (Widdowson and McCance, 1936; Sankaran and Rajagopul, 1938; M.R.C., 1943). However, O'Reilly (1948) and Garry *et al.* (1954) could not confirm this. The question of hormonal differences between these ethnic groups may well be important, but data are very scanty at the moment.

The Bungalow women's means for haemoglobin, red count and haematocrit do not differ significantly from the European values. This is at variance with my results at Haast's Bluff where the women's mean haemoglobin was almost as high as the men's. The result here conforms better to Walsh's (1957), Davis and Pitney's (1957), and Horsfall's (1957a) values of 13·2, 14·5 and 13·1 gm. Possible reasons for the high values found amongst the Haast's Bluff women are given in part I of this paper. Briefly, they were a high iron diet, low menstrual loss, and an underlying hormonal difference. The Bungalow women were still eating some native food, but were mainly on European rations. This means a reduction in the iron content of their diet. Nothing is known of their menstrual loss. However, when aborigines come to Adelaide their menstruation becomes more profuse and regular in place of their scanty, irregular loss in the bush. McArthur (1948), Wilson (1953) and Crotty (1958) record the nomadic aborigines' habit of breast feeding infants till 2 or 3 years of age. This would, to some extent, suppress menstruation.

The fact that red counts in this series were comparable with those of

Wintrobe (1956), and much greater than those reported for aborigines or Europeans in part I (Casley-Smith, 1958), implies that those results were too low, as was suspected. This was probably due to the unavoidable adsorption of some of the cells on to the bottles used. Still, the comparisons within the previous data remain valid.

Erythrocyte constants – M.C.V., M.C.H., M.C.H.C.

The mean corpuscular volume of the nomads was greater than Wintrobe's (1956) values for Europeans, but not significantly greater than Lancaster and Banks' (1957) values for Europeans in Sydney. The Bungalow group did not differ significantly from either the nomads or the Europeans. The increased M.C.V. of the nomads may reflect a greater erythropoiesis necessary to maintain their haemoglobins, as may also the increased anisocytosis and non-circularity (Casley-Smith, 1958).

The M.C.H's were all not significantly different from Wintrobe's figures, but were lower than Lancaster and Banks' values. Why these latter obtained their markedly higher values is uncertain, but is partly due to the use of iron content standardization in place of oxygen uptake methods. The high M.C.H.C's reflect the very good iron content of the natives' diets.

The M.C.H's of the nomads are greater than Wintrobe's values as might be anticipated from the M.C.V's, but values for the Bungalow men and women are not significantly different from his. However, these M.C.H's are all less than those found on the previous expedition, owing to the low red counts on that occasion. Also they are lower than Lancaster and Banks' values, probably because of new standards for the haemoglobin and the low red counts obtained of these workers.

Erythrocyte fragility.—There was quite a definite earlier start to haemolysis than in Europeans. However, the rest of the dilutions give approximately similar results. Differences in another ethnic group have been reported. Turner *et al.* (1937) found an increased resistance to haemolysis in negroes, as did Napier *et al.* (1941) for Indian women in Calcutta. The significance of these changes, if indeed they are real, can only be conjectured.

Erythrocyte sedimentation rate.—The E.S.R's of many groups have been investigated. Usually, the more primitive the conditions the greater are the rates. The results reported here fit in with these general trends, and with Horsfall's results (1957b) on Torres Strait Islanders. However, the natives in less contact with Europeans had lower values than those who lived near the town. This may reflect the poor resistance of the aborigine to European infection, and the fact that the squalor of a native settlement would provide good opportunities for the spread of diseases, e.g. the amount of tuberculosis in tribal aborigines is less than in urban ones (Woodruff, 1958).

It may be that the γ globulins produce these changes in the E.S.R. It is well known that γ globulins are high in aborigines (Curnow, 1957; Horsfall,

1957b; Brading, 1958; Wilkinson *et al.*, 1958). This again seems to be a feature common to most primitive communities. Another possible factor in raising the Bungalow groups' values may be their increased serum cholesterols when compared to the nomads' (Schwartz and Casley-Smith, 1958). But as these are both lower than European values, they do not account for the whole increase.

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THE EFFECT OF AMIPHENAZOLE ON MORPHINE-INDUCED ANALGESIA IN MICE

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SUMMARY.

The hot-plate method has been used on mice to investigate the effect of amiphenazole on the analgesic activity of morphine. Over a range of doses of each, morphine analgesia is potentiated by amiphenazole. The phenomenon has been observed with oral, intraperitoneal and subcutaneous routes of administration. In addition, morphine at a dose producing only slight analgesia potentiates the mild analgesic action of amiphenazole.

The effects of amiphenazole on codeine analgesia have been variable; at most, mixtures of the two drugs have shown a moderate but brief increase of analgesic effectiveness over that of the components given alone.

It is suggested that the morphine-potentiating property of amiphenazole may allow satisfactory clinical analgesia with doses of morphine less than those customary.

INTRODUCTION.

Since it was first observed to antagonise morphine-induced narcosis in dogs (Shaw and Bentley, 1952), amiphenazole (2:4-diamino-5-phenylthiazole, 'DAPTAZOLE') has been investigated extensively in both experimental and clinical pharmacology. In the dog it also antagonises the depressant effects of other morphine-like analgesics (Shaw and Bentley, 1955).

The ability of amiphenazole to prevent or reduce the stupefaction and respiratory depression produced by large doses of morphine is the basis for its clinical use, particularly in cases of terminal cancer (McKeogh and Shaw, 1956). Its effect on the analgesic properties of morphine is therefore of importance. Clinical observers have found consistently that amiphenazole exerts no antagonism against the analgesia produced by morphine and allied drugs (Shaw and Shulman, 1955a, 1955b; O'Connell, 1956; Holmes, 1956; Dundee, 1957). However, in conflict with this clinical experience, Mercier (1956) has reported that amiphenazole interferes with the analgesic effect of morphine on thermal pain thresholds in mice.

Preliminary experiments in this laboratory suggested that rather than antagonising morphine analgesia in mice, amiphenazole potentiated it. Further-

more, amiphenazole appeared to possess analgesic properties of its own. The present paper contains the results of a more detailed investigation of the interaction between amiphenazole and morphine as analgesic substances in mice. In addition, the results of some experiments using codeine with amiphenazole are presented.

Since the experiments were completed, the author has seen a report that amiphenazole intensifies morphine analgesia in rats (Kruglov, 1957).

Methods.

Measurement of analgesia.

The "hot-plate" method (Eddy *et al.*, 1950, 1953) was used for measuring analgesia. Instead of a glass cylinder, a shallow perspex enclosure with a removable lid was placed on the hot surface. It was found that boiling acetone alone held the temperature at 55° C. better than Eddy's acetone-ethyl formate mixture.

The reaction taken for latency measurements was a characteristic operation by the mouse on both fore-paws simultaneously. Because of its rapidity, observers were unable to agree whether it was a licking, blowing, or wiping action. In any case, its appearance was found to be more reliable than the other varieties of reaction (jumping, shaking of hind-legs, "dancing") used in combination by Eddy. On some occasions, there would be mice which, instead of reacting with the fore-paws, engaged in persistent though futile escape manoeuvres (jumping against lid, clawing at corners of the enclosure). The latency for initiation of such activity was then taken as reaction-time, if necessary the estimation being repeated after an interval of some minutes. The time between placing a mouse on the plate and occurrence of the chosen reaction was measured to the nearest second. The mouse was then removed as quickly as possible. If an animal did not respond within 60 seconds its reaction-time was scored as "greater than 60". If a mouse micturated while on the plate, the reading was interrupted, the plate cleaned, and the estimation repeated after an interval of at least five minutes. For each mouse, an analgesic increment was the difference between its reaction times before and after dosage. For the former figure, the mean of two pre-dosage times was used. Reaction-times were measured at 15, 75 and 135 minutes after dosage.

In each experiment, dosage-groups of 6 to 10 mice were used. A control group receiving saline only was used. Mice were allotted to groups by a process of randomization restricted so as to avoid difference due to pre-dosage reaction-times. They were then given the appropriate drugs in such an order that one mouse was selected from each group in turn, and the cycle repeated until all mice were dosed. Reaction-times after dosage were determined in the same order.

The mice were of a hybrid strain bred in the laboratory. Male animals only, weighing 25-45 gm., were used. There was no systematic variation in reaction-time with body-weight.

Routes of administration.

In most experiments drugs were given by the oral route. A few experiments were conducted using intraperitoneal and subcutaneous administration.

Treatment of data.

The mean analgesic effect in a group was taken as the difference between its mean analgesic increment and that of the control group. For the purposes of analysis, a reaction time greater than 60 sec. was taken as meaning an increment of 60, despite the resulting bias.

Dose-response relationships were investigated by the conventional techniques of regression analysis (Burn *et al.*, 1950). When a dose-response line was estimated more than once, the slopes were pooled when statistically permissible (95 p.c. Confidence level).

Drugs.

The drugs used were: morphine hydrochloride, amiphenazole hydrochloride and codeine phosphate. They were dissolved in distilled water for administration. Concentrations were adjusted so that each mouse received a volume of 0·1 ml. per 10 gm. body weight. For combined dosage, two drugs were dissolved in the same solution.

Assessment of interaction.

The effect of amiphenazole on morphine-induced analgesia was examined in several dose-combinations. The evidence for interaction derives from: the separate effects of morphine and amiphenazole compared with that of combinations of the two; the effect of a fixed dose of amiphenazole when added to a graded series of morphine doses; and the effect of increasing doses of amiphenazole added to a fixed dose of morphine. The latter two effects were tested only by the oral route. In a few experiments the combination of codeine and amiphenazole was compared with the individual drugs.

In a study of drug interactions it can be difficult to distinguish potentiation from mere addition, especially when the drugs are used at doses around their threshold levels. In order to avoid this difficulty all mean analgesic effects as defined above were translated into dose-equivalents of morphine, by the following method.

A straight line of slope determined in other experiments (see above) was drawn through the point representing the mean analgesic effect of morphine at the dose used in the given experiment. From this graph the doses of morphine were read off which would give mean effects equal to those obtained respectively from amiphenazole and the morphine-amiphenazole combination. The morphine doses so obtained from the graph were designated the "morphine-equivalents" of amiphenazole or of the combination. All conclusions regarding the nature of drug interaction derive from the relative magnitudes of such morphine-equivalents.

It is convenient to express these procedures in symbolic form. Let M be the dose of morphine, A be the dose of amiphenazole, M_A be the morphine-equivalent of A obtained by reference to the morphine dose-response relationship, and M_{MA} be the corresponding morphine-equivalent of the combination of doses: M + A. Then if amiphenazole antagonized morphine analgesia, the morphine-equivalent (M_{MA}) would be less than the dose of morphine given (M). If amiphenazole potentiated morphine, the morphine-equivalent of the combination would exceed the sum ($M + M_A$). If the two drugs were additive in effect without potentiation, then M_{MA} would be greater than M but less than or equal to $M + M_A$. Thus, the relations which serve in this paper as criteria for the nature of the interaction are as follows:

Antagonism of morphine analgesia	$M_{MA} < M$
No effect on morphine analgesia	$M_{MA} = M$
Addition with occlusion of effects	$M < M_{MA} < M + M_A$
Simple addition of effects	$M_{MA} = M + M_A$
Potentiation of morphine analgesia	$M_{MA} > M + M_A$

However, since morphine was much more potent an analgesic than amiphenazole, in terms both of the doses required to achieve moderate analgesia, and of the degree of analgesia obtainable as doses were increased, it was thought that a more descriptive if less valid expression for the effect of the combinations would be given by a "potentiation" factor, *viz.* the ratio of morphine-equivalent of combination to actual dose of morphine, or M_{MA}/M.

In tabulating the results, "potentiation" factors of morphine were calculated as well as the magnitudes in terms of morphine dosage, $M_{MA} - (M + M_A)$, by which the combination of morphine and amiphenazole surpassed simple addition of analgesic effects.

In supplementary experiments the slope of the dose-response regression line for morphine

when combined with a fixed dose of amiphenazole was compared with the previously established slope for morphine alone. Conversely, the dose-response relationship for amiphenazole when combined with a fixed dose of morphine was compared with those for morphine and amiphenazole alone, as determined in separate experiments.

RESULTS.

Dose-response relationships for morphine, amiphenazole and codeine.

Since in most experiments drugs were given by oral route, more attention was paid to the estimation of dose-response relations by this route. With morphine, 20 mg./kg. was usually near the threshold dose-level for analgesia measured at 15 and 75 minutes, and the arbitrary maximum of 60 seconds increment was approached at doses above 160 mg./kg. At the 135-minute period, analgesic effects of morphine were shown only at the upper end of this dose-range.

On sub-cutaneous administration, morphine exhibited about five times the oral potency. The comparable dose-range was 4-40 mg./kg. Intraperitoneal potency lay between the oral and subcutaneous, with appropriate dose-range of approximately 10-80 mg./kg. With both parenteral routes, analgesia was observed at 135 minutes only at the higher doses.

The analgesic response varied linearly with dose, although doses were at first spaced in expectation of a regression on log dose. The regression equations fitted to the results in separate estimations, and those obtained by pooling these estimates when shown permissible by analysis of variance, are shown in Table 1, together with the standard errors of the estimated regression coefficients.

Amiphenazole was much less potent as an analgesic than morphine by all routes. No single estimation gave a satisfactory regression on oral dosage. Finally, all observed mean points were pooled, giving a significant regression for the 15 minutes observation time, but not for 75 minutes, nor for the average of 15, 75 and 135 minutes. As shown in Table 2, the analgesic effect of amiphenazole fell off sharply between 15 and 75 minutes on parenteral administration also. The dose-ranges for amiphenazole analgesia were: for the oral route, minimal analgesia up to about 100 mg./kg., increasing to a moderate level at about 300 mg./kg.; for subcutaneous and intraperitoneal dosage, steady increase from threshold to moderate levels over the range 30 to 200 mg./kg. By all routes, analgesic effects had disappeared by 135 minutes, and were small even with the highest doses at 75 minutes.

The regression, when significant, was linear on dose.

With codeine it was found impossible to obtain satisfactory regression relationships by the oral route. Mean analgesia remained at a low level up to about 150 mg./kg., then increased to moderate levels at about 250 mg./kg. However, the variability of response was large, and in some estimates bell-shaped dose-response curves appeared. Codeine was not given by parenteral routes.

TABLE 1.

Dose-response regression relationships for morphine.

Route / Time after dosage	Replication	Regression equations			Standard error of regression coefficient		
		Oral	Sub-cut.	Intraperit.	Oral	S.-cut.	I.P.
15 minutes	(a)	$y-16=0\cdot24\ (x-75)$	$y-45=1\cdot2\ (x-17)$	$y-42=1\cdot4\ (x-30)$	0·062	0·38	0·38
	(b)	$y-25=0\cdot32\ (x-75)$	$y-15=1\cdot0\ (x-15)$	$y-28=1\cdot6\ (x-23)$	0·081	0·44	0·37
	(c)	$y-11=0\cdot25\ (x-75)$			0·088		
	Av.	$y-20=0\cdot27\ (x-75)$	$y-\bar{y}=1\cdot1\ (x-\bar{x})$*	$y-36=1\cdot5\ (x-27)$	0·042	0·30	0·26
75 minutes	(a)	$y-18=0\cdot33\ (x-75)$	$y-29=1\cdot8\ (x-17)$	$y-41=0\cdot66\ (x-45)$	0·045	0·38	0·17
	(b)	$y-25=0\cdot31\ (x-75)$	$y-22=2\cdot1\ (x-19)$	$y-26=0\cdot76\ (x-47)$	0·084	0·39	0·20
	(c)	**					
	Av.	$y-20=0\cdot32\ (x-75)$	$y-29=1\cdot9\ (x-18)$	$y-\bar{y}=0\cdot69\ (x-\bar{x})$*	0·041	0·28	0·13
Average of 15, 75, 135 min.	(a)	$y-17=0\cdot30\ (x-75)$	$y-36=0\cdot72\ (x-29)$	$y-18=0\cdot53\ (x-38)$†	0·044	0·10	0·11
	(b)	$y-23=0\cdot22\ (x-93)$	$y-16=1\cdot1\ (x-19)$		0·097	0·34	
	(c)	$y-8=0\cdot16\ (x-93)$			0·066		
	Av.	$y-17=0\cdot26\ (x-75)$	$y-32=0\cdot76\ (x-26)$		0·031	0·095	

* Slopes only pooled: Significant differences of position.

** The 75-minute regression for this replication differed significantly from (a) and (b), and therefore was not included in the pooled average.

† Linear regression in only one of two trials.

In all lines shown, deviations from linearity insignificant at 95 p.c. confidence level. Pooled slopes and pooled positions differ insignificantly at 95 p.c. level.

y = Analgesic effect.

x = Dose (mg./kg.).

TABLE 2.

Dose-response regression relationships for amiphenazole.

Route / Time after dose	Regression equations			Standard error of regression coefficient		
	Oral	Sub-cut.	Intraperit.	Oral	S.-cut.	I.P.
15 min.	(Pooled)* $y-13=0\cdot07\ (x-122)$	$y-13=0\cdot14\ (x-94)$	$y-9=0\cdot19\ (x-56)$	0·023	0·043	0·040
75 min.	$y-4=0\cdot02\ (x-122)$ (N.S.)	$y-4=0\cdot02\ (x-94)$ (N.S.)	$y=0\cdot06\ (x-56)$	0·03	0·02	0·022
Average of 15, 75, 135 min.	$y-4=0\cdot02\ (x-122)$ (N.S.)	$y-5=0\cdot05\ (x-94)$	$y-4=0\cdot13\ (x-56)$	0·02	0·18	0·030

y = Analgesic effect.
x = Dose (mg./kg.).
* All observed mean analgesias pooled in calculation of oral regression equations.
N.S. Regressions not significant.

TABLE 3.

Effect of amiphenazole on morphine analgesia in mice. Oral administration.

Dosage (mg./kg.)		Mean analgesic effect of			"Potentiation" factor for morphine	Morphine equivalent of combination *minus* (Morphine equivalent of A plus M)
AMPH (A)	MORPH (M)	AMPH (E_A)	MORPH (E_M)	AMPH + MORPH (E_{MA})	$\frac{M_{MA}}{M}$	$M_{MA}-(M_A+M)$
				15 minutes after dosage		
25	30	—	−1	6	2·0	—
50	20	−1	−1	22_{++}**	5·0	65
50	30	13	6	24*	3·2	11
50	30	—	−1	29**	5·0	—
50	50	8	15	43**	3·0	79
50	75	0	25**	51_{+}**	2·4	100
100	15	9*	—	22**	5·9	33
100	30	—	−1	55**	>7	—
100	30	9*	—	37**	5·2	86
100	60	9*	—	53**	3·6	100
100	75	12**	25*	52**	2·4	75
200	60	14*	36**	44**	1·5	27
				75 minutes after dosage		
25	30	—	−2	2	1·3	—
50	20	−1	−2	11_{+}	3·0	15
50	30	5	2	17*	2·6	10
50	30	—	−2	20**	2·3	—
50	50	6	8	29**	2·3	21
50	75	−7	9	31_{+}**	1·9	41
100	15	0	—	15 N.S.	4·5	26
100	30	—	−2	11	3·3	—
100	30	0	—	16*	2·5	19
100	60	0	—	41**	2·6	68
100	75	15*	10**	35**	2·2	−13
200	60	13	13	20	1·4	−37
				Average of effects at 15, 75 and 135 minutes after dosage		
25	30	—	−2	2	1·5	—
50	20	−1	0	14_{+}*	3·5	35
50	30	8	4	16*	2·6	4
50	30	—	−2	16**	3·3	—
50	50	6	10	30**	2·6	45
50	75	−3	15*	31_{+}**	1·9	58
100	15	−2	—	13*	4·7	43
100	30	—	−2	24**	4·3	—
100	30	−2	—	22**	3·5	63
100	60	−2	—	38**	2·8	95
100	75	10*	11*	30**	1·9	0
200	60	9	20*	24**	1·3	4

Significant at * 95 p.c. / ** 99 p.c. } levels of confidence.

Difference from sum: (E_A+E_M) sig. at: $_{+}$95 p.c. / $_{++}$99 p.c. } levels of confidence.

M_A = Morphine-equivalent of amiphenazole at dose (A).

M_{MA} = Morphine-equivalent of morphine-amiphenazole combination.

Analgesic effects of morphine-amiphenazole mixtures.

In Tables 3, 4 and 5 the results obtained after simultaneous administration of morphine and amiphenazole are summarised.

The most striking result from the oral tests was that doses of morphine and amiphenazole which singly gave insignificant degrees of analgesia could produce a marked effect in combination (Table 3). The figures entered under "Potentiation Factor" indicate the degrees by which the apparent potency of morphine was increased by amiphenazole. Evidence for true potentiation, above mere addition, is contained in the last column where the criterion for a potentiating interaction adopted here is satisfied for most of the dosage-combinations used. The means of the figures in this column differ from zero with a high degree of significance ($P < 0{\cdot}01$ in t-test) at 15 minutes and in the average for the three observation-times, but not at 75 minutes. If the experiment using amiphenazole at 200 mg./kg. be excluded, the mean potentiation for the remaining figures is significant at 75 minutes also ($P < 0{\cdot}05$).

TABLE 4.

Effect of amiphenazole on morphine analgesia in mice. Subcutaneous administration.

Dosage (mg./kg.)		Mean analgesic effect of			"Potentiation" factor for morphine	Morphine equivalent of combination *minus* (morphine equivalent of A plus M)
AMPH (A)	MORPH (M)	AMPH (E_A)	MORPH (E_M)	AMPH + MORPH (E_{MA})	$\frac{M_{MA}}{M}$	$M_{MA}-(M_A+M)$
			15 minutes after dosage			
10	5	0	9*	8**	1	0
20	5	2	7**	15**	2·6	7·5
50	20	11**	38**	52**	1·7	14
			75 minutes after dosage			
10	5	0	11	4	0·3	0
20	5	−4	6	4	0·7	0
50	20	1	30*	56_{++}**	1·7	8
			Averages of effects at 15, 75 and 135 minutes after dosage			
10	5	1	8*	4	0·5	0
20	5	−1	4	6*	1·5	0
50	20	6*	27**	37*	1·7	13

Significant at * 95 p.c. ** 99 p.c. } levels of confidence.

Difference from sum: (E_A+E_M) sig. at: $_{+}$95 p.c. $_{++}$99 p.c. } levels of confidence.

M_A = Morphine-equivalent of amiphenazole at dose (A).

M_{MA} = Morphine-equivalent of morphine-amiphenazole combination.

The results for subcutaneous (Table 4) and intraperitoneal (Table 5) dosage are less extensive, but show that using suitable dose-levels potentiating effects could be obtained as with the oral route.

TABLE 5.

Effect of amiphenazole on morphine analgesia in mice. Intraperitoneal administration.

Dosage (mg./kg.)		Mean analgesic effect of			"Potentiation" factor for morphine	Morphine equivalent of combination *minus* (morphine equivalent of A plus M)
AMPH (A)	MORPH (M)	AMPH (E_A)	MORPH (E_M)	AMPH + MORPH (E_{MA})	$\frac{M_{MA}}{M}$	$M_{MA}-(M_A+M)$
			15 minutes after dosage			
50	15	6*	19**	38**	1·8	5
50	20	19*	23*	48**	1·8	0
			75 minutes after dosage			
50	15	9*	6	36_+**	3·8	23
50	20	1	6	32_+**	2·9	23
			Average of effects at 15, 75 and 135 minutes after dosage			
50	15	9	8	27**	3·6	20
50	20	8*	11*	29**	2·7	20

Significant at * 95 p.c. ** 99 p.c. } levels of confidence.
Difference from sum: (E_A+E_M) sig. at: $_+$95 p.c. $_{++}$99 p.c. } levels of confidence.
M_A = Morphine-equivalent of amiphenazole at dose (A).
M_{MA} = Morphine-equivalent of morphine-amiphenazole combination.

TABLE 6.

Regression coefficients for dose-response relationships of amiphenazole and morphine each alone and in the presence of a poorly active dose of the other. Oral route.

Time after dosage	Regression coefficients and equations				Regression ratios	
	Morph. b_M	Amph. b_A	Morphine + Amph. 100 mg./kg. $b_{M(A)}$	Amiphenazole + Morph. 30 mg./kg. $b_{A(M)}$	$\frac{b_{M(A)}}{b_M}$	$\frac{b_{A(M)}}{b_A}$
15 min.	0·267	0·07	0·646 $y-37·4=0·646(x-34·95)$	0·623 $y-29·8=0·623(x-58·3)$	2·4	8·9
75 min.	0·324	0·02 (N.S.)	0·606 $y-23·8=0·606(x-34·95)$	0·07 (N.S.)	1·9	(3·5)
Average of 15, 75, 135 min.	0·259	0·02 (N.S.)	0·556 $y-24·2=0·556(x-34·95)$	0·265 $y-14·0=0·265(x-58·3)$	2·1	(13)

No experiments were performed in which morphine was administered by one route and amiphenazole by another.

In Table 6 are shown the effects of amiphenazole at a single dose on the regression coefficient for morphine analgesia, and of a single dose of morphine on that for amiphenazole analgesia, by oral administration. The data from these experiments are included in Table 3 also.

Codeine-amiphenazole mixtures.

In one experiment codeine and amiphenazole were each inactive at a dose of 50 mg./kg. *per os*, but the combined drugs at the same dose produced a low level of analgesia. Amiphenazole alone at 100 mg./kg. gave a minimal analgesic effect, but when it was combined with codeine at 50 mg./kg. the analgesic score was increased several-fold. This increase was confined to the reading at 15 minutes (Table 7). In another experiment not tabulated codeine at 100 mg./kg. had a mild analgesic effect. Amiphenazole produced no increment in analgesia at either 50 or 100 mg./kg.

TABLE 7.

Analgesic effects of codeine-amiphenazole mixtures. Oral administration.

Treatment	Mean analgesia at:			
	15 min.	75 min.	135 min.	Average of 3 times
Codeine 50 mg./kg.	3	−1	− 1	0
Amiphenazole 50 mg./kg.	0	−1	0	0
Amiphenazole 100 mg./kg.	3	8	9	6
Codeine 50 mg./kg. *plus* Amiphenazole 50 mg./kg.	11	8	12	10
Codeine 50 mg./kg. *plus* Amiphenazole 100 mg./kg.	39**	9	11	20*

* Significantly greater than codeine 50 mg./kg. ($0{\cdot}01 < P < 0{\cdot}05$).
** Significantly greater than codeine 50 mg./kg. or amiphenazole 100 mg./kg. alone. ($P < 0{\cdot}01$).

Codeine at 200 mg./kg. *per os* gave moderate analgesia. In groups which in addition received amiphenazole at 50 and 100 mg./kg. there were 50 p.c. and 100 p.c. deaths respectively.

DISCUSSION.

The hot-plate method suffers from the disadvantage common to most methods for testing analgesia in animals, that reaction-time does not necessarily bear a simple relationship to pain sensation. Further, the reaction of mice on the hot-plate is a highly coordinated motor act, so that false "analgesic" scores could occur if a drug interfered with effector mechanisms. In the present investigation, mice given higher doses of morphine were noticeably less active,

which makes it possible that increased reaction-times were due to general sedation as well as analgesia.

A high variability of reaction-times was encountered, and this depended partly on operational difficulty in detecting the chosen end-point, particularly when mice had already undergone several trials during an experiment. This difficulty would probably be reduced by using different groups of mice for the estimation of analgesia at each time-interval. However, the variability was not wholly operational but partly drug-dependent, since it was greater with codeine than with morphine.

The present investigation has been limited mainly to the effect of a weak analgesic (amiphenazole) on the activity of a strong one (morphine). The method of analysis adopted, in which a dose of amiphenazole is expressed as a dose of morphine of equivalent activity, depends on the assumption that the pooled regression-coefficients for morphine adequately represent the particular cases where amiphenazole-morphine combinations are compared with a single dose of morphine. The data in Table 1 largely justify this assumption, since in most instances separate estimations of slope for morphine analgesia did not differ significantly at the 95 p.c. confidence level.

When the modes and even sites of action of two drugs are unknown, discussion of their pharmacological interactions in terms of potentiation or antagonism lacks precise meaning. The "isobol" method (Loewe, 1957) enables such interactions to be studied without using these concepts. In the present investigation, however, both drugs alone were analgesic, and it seemed reasonable that antagonism was definable as reduction of the effect of the stronger drug when the weaker was added to it. On the other hand the criterion used for potentiation was somewhat arbitrary. But since amiphenazole has considerably less analgesic potency than morphine, the procedure of translating its doses into equieffective doses of morphine, adding them to the actual dose of morphine and finally comparing the sum with the dose of morphine estimated to be equivalent in effect to the combination of the two drugs, probably results in relative over-estimation of the effect which would be expected from mere addition. Conclusions that "potentiation", as defined here, occurred can therefore be regarded as conservative.

The present results are consistent with those obtained by Kruglov (1957), who used reaction-times to thermal irradiation of the rat's tail. His data show that amiphenazole does not antagonise the analgesic action of morphine, but reinforces it. He gave morphine and amiphenazole by the intraperitoneal route only, one drug following the other after an interval of 30-60 minutes, and followed the time-course of caudal reaction-latency. He found that 5 mg./kg. morphine produced a secondary rise in reaction-time when injected after 50 mg./kg. amiphenazole and vice versa. Since control data for each drug alone, and for untreated animals, are not supplied, it is impossible to judge whether

the analgesic effect of morphine was intensified, prolonged or merely supplemented.

Mercier's (1956) data appear to contradict the potentiation of morphine by amiphenazole. He found that amiphenazole injected subcutaneously into mice at 2 or 4 mg./kg. with morphine at 10 mg./kg. antagonised the analgesic action of the latter. The doses of morphine he used are at the lower end of the analgesic range found in the present experiments, and the doses of amiphenazole are far below those capable of giving analgesia. Inspection of Table 4 above suggests a slight antagonism when small doses of amiphenazole and morphine are given subcutaneously. However, with higher doses there is a definite and significant potentiation, although less pronounced than with other routes of injection. The discrepancies between Mercier's data and my own may thus depend on difference in dose-ratios.

The analgesic potency of amiphenazole relative to morphine cannot be estimated precisely from the present results. In general, the comparable regression lines for each drug do not fulfil the criteria for a valid slope-ratio comparison (Burn *et al.*, 1950). Even if these are ignored, the resulting potency-estimates vary widely with routes of administration and times of observation. An average figure of about one-tenth could be given for the relative potency of amiphenazole with variation by a factor of two in either direction under the different conditions of observation used.

The data in Table 6 leave no doubt that with the present method amiphenazole approximately doubles the analgesic potency of morphine, and that for the 15-minute observation-time at least, morphine produces nearly a tenfold increase in analgesic potency of amiphenazole. It is perhaps of more interest that doses of amiphenazole and morphine, alone ineffective, could when combined give rise to considerable levels of analgesia. This observation is limited to the results of oral administration, for which there is fullest information.

The present results neither support nor refute the hypothesis advanced by Gershon *et al.* (1958) that a new analgesic substance is formed from morphine and amiphenazole in the liver. Potentiation of morphine analgesia was usually greatest at the shortest interval of 15 minutes after dosage; this is possibly too soon to be attributed to a product of intrahepatic metabolism.

No clinical observations have been published in which barely-effective doses of amiphenazole and morphine have been given in combination. So far amiphenazole has been used mainly to allow safer extension of the morphine dosage to high levels. The present data suggest another approach to the clinical use of amiphenazole. It may be possible to attain satisfactory levels of analgesia with doses of morphine much lower than usual by conjoint administration of amiphenazole. The value of this suggestion depends not only on the pertinence to clinical conditions of the results with mice, but also on the question of toxicity. Amiphenazole, at doses which partially antagonize the respiratory

inhibition by morphine in rabbits, almost doubles the acute toxicity of the drug to mice (Kruglov, 1957; Mercier, 1956). If this increase in acute toxicity were reflected in chronic toxic effects when lower doses were given repeatedly, the clinical use of the combined drugs as suggested could not be undertaken with safety. Experiments to investigate this question are in progress at this laboratory.

The results with codeine and amiphenazole are not extensive enough to allow firm conclusions, and failure to obtain satisfactory dose-response relationships with codeine prohibits treatment of the results as for morphine. Any potentiation observed was limited to the 15-minute period, and attempts to extend its duration and intensity by increasing the dose of codeine did not succeed. In fact, the greater toxicity of the mixture became evident at dose-levels quite moderate for each drug alone.

Although codeine differs only slightly in chemical structure from morphine (having a methoxyl instead of a phenolic hydroxyl group), its analgesic potency is much inferior. The virtual inability of amiphenazole to potentiate codeine analgesia suggests that the interaction between amiphenazole and morphine similarly depends on some specificity of chemical structure, perhaps related to the presence of the phenolic hydroxyl group.

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THE EFFECT OF HYDROGEN ION CONCENTRATION AND OF HEAT ON THE COMPLEMENT FIXING ANTIGEN AND HAEMAGGLUTININ OF ECTROMELIA VIRUS

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SUMMARY.

Both the haemagglutinin and the complement fixing antigen of ectromelia virus are fairly stable at hydrogen ion concentrations ranging from pH 4 or 5 to 11, although the highest titres were found around the neutral point.

Ectromelia complement fixing antigen showed some drop in titre on heating at 56° C. for 30 minutes, but no more so than vaccinial preparations run in parallel.

The haemagglutinin seems to have a heat labile as well as a heat stable component. The heat labile component was neutralised by rabbit anti-serum but not by normal rabbit serum and so appears to be specific.

INTRODUCTION.

The classical work of Craigie and Wishart (1936, a and b) on the soluble antigen of vaccinia virus (which seems to be identical with the complement fixing antigen) revealed the existence of a heat labile (L) and a heat stable (S) component which seemed to occur in intimate association (LS) on the surface of the elementary body as well as in solution free from virus. Shedlovsky and Smadel (1942) succeeded in isolating the pure LS protein and showed that it was a single molecule that was capable of eliciting two distinct antibodies. The heated antigen would not give a positive precipitin reaction with L antibody but would still inhibit its action. If the antigen was heated with alkali it would no longer inhibit L antibody nor precipitate with S antibody, although it would still inhibit the latter. On the other hand, Mayr, Herrlich and Mahnel (1955) considered that the so-called L component was identical with a host specific normal component that they found only in their crude antigen and which was removed during purification of the soluble substance. Their final antigen withstood boiling for five minutes and was very stable on storage. Apparently they obtained similar results with ectromelia, cowpox and fowlpox viruses. MacDonald and Downie (1950) also studied the effect of heat on the complement fixing titres of the pox viruses including ectromelia and vaccinia and found that the titre was reduced by ¼ to ½ on heating at 68-70° C. for an hour.

Chu (1948) reported that the vaccinia haemagglutinin was a remarkably thermostable substance since it resisted a temperature of 98-100° C. for 10 minutes although it was destroyed by autoclaving at 15 lb. for 20 minutes. Some batches prepared from infected chorio-allantoic membranes showed a drop in haemagglutinin titre after heating to 56° C. and no further drop when heated at higher temperatures, but Chu concluded that there was no adequate evidence for the existence of thermostable and thermolabile components. Mayr (1956) also considered that the specific haemagglutinin of vaccinia virus, as well as those of ectromelia, cowpox and fowlpox viruses, was thermostable (56° C. for 30 minutes) and that any thermolabile haemagglutinin was the same as the non-specific haemagglutinin that could be isolated from uninfected chorio-allantoic membranes. However, Gillen, Burr and Nagler (1950) thought that vaccinial haemagglutinin had two components which could be separated by high speed centrifugation and that that in the deposit was heat stable while that in the supernate was much more labile.

The effect of hydrogen ion concentration on the antigens of the pox viruses has not been studied to the same extent as the effect of heat but it seems that the antigens are fairly stable to changes in pH. (cf. Smadel and Hoagland, 1942; Chu, 1948).

The object of this work was to see whether the antigens of ectromelia virus behaved in the same way as those of the much studied vaccinia virus, the ultimate aim being to devise a method for purifying and concentrating the antigens of ectromelia.

Materials and Methods.

Virus strains.

Ectromelia. The Hampstead (egg passaged) strain (cf. Fenner, 1949).

Vaccinia. Strain Gillard, which was derived from a commercial vaccinial lymph and has since been passaged frequently in rabbits. Received from the Walter and Eliza Hall institute.

Virus and haemagglutinin suspensions.

Virus and other antigens were recovered from infected chorio-allantoic membranes (after three days' incubation for ectromelia virus and two days for vaccinia virus) by homogenising with saline in a mortar pre-chilled to — 20° C. or in an MSE tissue homogeniser. The homogenates were rather concentrated, ranging from 20 p.c. W/V to 50 p.c. W/V, and were clarified by centrifuging at 600 g. for 10 minutes in an angle centrifuge (= original suspension). In some cases this suspension was spun for 30 minutes at 10,000 r.p.m. in the rotor 40 of a Spinco model L centrifuge ($= 2 \times 10^5$ g. minutes) which reduced the virus content to about 1 p.c. of the original but still left a lot of haemagglutinin in the supernate. Finally, most of the haemagglutinin could be spun down by centrifuging for 5×10^6 g. minutes (35,000 r.p.m. for one hour in rotor 40) and resuspended in saline (= "haemagglutinin" suspension). The supernate represented the "soluble" antigen and had an infective titre of about 0·01 p.c. of the original.

Antisera.

Antisera against vaccinia virus were prepared on the lines laid down by Craigie and Wishart (1934). Antisera against ectromelia virus were prepared in rabbits by the intravenous injection of the washed deposit of a homogenate of infected mouse liver centrifuged for 180,000 g. minutes after a preliminary centrifugation for 6,000 g. minutes. Convalescent mouse serum was also used.

Virus titrations.

The viruses were titrated in eggs using the pock counting technique described by Beveridge and Burnet (1946).

Haemagglutination test.

This was based on the procedure described by Chu (1948), using 0·5 p.c. fowl cells. Cells and haemagglutinin were allowed to react for 10 minutes at 35° C., shaken and then re-incubated for 30 minutes before reading. The diluent was either saline or McIlvaine's buffer (pH 7·2) diluted 1 in 50 in saline, to which 5 p.c. inactivated rabbit serum was added: the addition of normal rabbit serum minimises false positive reactions.

Complement fixation test.

The technique adopted was that of Donnelley (1951).

EXPERIMENTAL.

The effect of pH.

The method employed was similar to that used by Chu (1948) in his studies on vaccinia haemagglutinin, and covered the range pH 2-12 in steps of 1·0. McIlvaine's citrate phosphate buffer was used at pH 2·0-8·0, Sørensen's borate HCl and Gomori's (1946) 0·05M tris HCl buffers at pH 9·0, Sørensen's borate NaOH buffer at pH 10·0, and Ringer's phosphate NaOH buffer at pH 11·0 and 12·0. In addition, Sørensen's glycine NaOH was used at pH 9·0 and 10·0. Two kinds of antigen suspension, prepared as set out in the materials and methods, the original suspension and the "haemagglutinin" suspension, were brought to the desired pH by adding 0·5 ml. to 2 ml. of buffer after which the pH was checked by means of the glass electrode and brought to the required pH, if necessary, by the addition of the appropriate member of the buffer pair. The mixtures were then left for about 20 hours at 2° C. when the pH of each tube was brought to about 7·0 and the volume made up to 5 ml. with physiological saline. A precipitate formed at pH 2·0, 3·0 and 4·0 in those tubes containing the "haemagglutinin" suspension and at pH 3·0, 4·0 and sometimes 5·0 in those with the original suspension. This precipitate seemed to go partly into solution when the pH was brought to 7·0; it became very fine and remained in suspension for some hours, so no attempt was made to remove it before carrying out the tests.

The haemagglutinin titre of each sample was determined using rabbit serum buffered saline as the diluent. In addition, each sample was tested for its power to fix complement in the presence of 1/20 antivaccinial rabbit hyper-

TABLE 1.

Effect of pH on the titre of ectromelia complement-fixing antigen and haemagglutinin. Exposed for 20 hours at 2° C.

	pH	2	3	4	5	6	7	8	9	10	11	12
Complement fixing titre of												
1. Original suspension		30	30	40	60	75	75	60	40	40	40	40
2. "Haemagglutinin" suspension		<10	<10	15	15	20	40	35	30	30	30	40
Haemagglutinin titre of												
1. Original suspension		40	15	40	160	160	240	160	60	80	60	10
2. "Haemagglutinin" suspension		30	30	30	120	240	240	120	80	40	60	10

immune serum. A representative series of results are set out in Table 1. They indicate that the complement fixing antigen is stable over a considerable pH range, although the titres were highest from pH 6·0 to 8·0. Only in the case of the "haemagglutinin" suspension at pH 2·0 and 3·0 was there a marked fall in titre (to less than one-quarter of the maximum). The haemagglutinin was also very stable: the titres did not differ significantly over the range pH 5·0-8·0. Outside this range the titre fell slightly; perhaps more destruction of haemagglutinin occurred on the acid than on the alkaline side.

The effect of heat.

The antigen suspensions, which were prepared as detailed above, were usually heated at 56° C. for 30 minutes, but on a few occasions the effect of 68-70° C. for 30 minutes was also tested. The effect on the complement fixing antigen is shown in Table 2, and the effect on the haemagglutinin in Table 3. An antivaccinial rabbit hyperimmune serum was used to give the results of Table 2, but similar results were obtained when using anti-ectromelia rabbit immune serum. However, a mouse immune serum which had a low titre (1/75) with the unheated antigen was negative with the lowest dilution of the heated

TABLE 2.

The effect of heat on the complement-fixing antigen of ectromelia virus. Titres are expressed per gram wet weight of tissue.

	Centrifugal force × time g. minutes	Supernate		Deposit		Original suspension	
		unheated	heated	unheated	heated	unheated	heated
Experiment 1							
Ectromelia	$2{\cdot}2\times10^5$	300	+100	150	100	600	300
Vaccinia	$2{\cdot}2\times10^5$	1,600	+1,200	800	400	2,400	800
Experiment 2							
Ectromelia	$5{\cdot}0\times10^6$	80	30	130	50	320	240

+Heated at 68-70° C. for 30 minutes. The other antigens were heated at 56° C. for 30 minutes.

TABLE 3.

The effect of heat on the haemagglutinin of ectromelia virus.

	Centrifugal force × time g. minutes	Supernate			Deposit			Original suspension		
			heated 30 min.			heated 30 min.			heated 30 min.	
		unheated	56° C.	68-70° C.	unheated	56° C.	68-70° C.	unheated	56° C.	68-70° C.
Experiment 1										
Ectromelia	$2{\cdot}2\times10^5$	120	60	5	80	40	—	320	60	—
Vaccinia	$2{\cdot}2\times10^5$	320	120	<5	160	60	—	640	60	—
Experiment 2										
Ectromelia	$5{\cdot}0\times10^6$	—	—	—	5,120	640	320	1,280	160	60
		Washed deposit								
Experiment 3										
Ectromelia	$5{\cdot}0\times10^6$	1,280	960	320	3,840	1,280	640	480	120	60

Since the antigen was concentrated in certain cases the titres have not been converted to a basis of grams wet weight of tissue.

antigen that was tested (1/25). The "washed deposit" of Table 3 was prepared by three cycles of low and high speed centrifugation using 1/50 McIlvaine's buffer as the washing fluid.

DISCUSSION.

Chu (1948) found that vaccinia haemagglutinin was stable over the range pH 5·9 to 9·8 and that the titre fell off more markedly on the acid than on the alkaline side. Tagaya (1954a) obtained similar results, although in this case the haemagglutinin was exposed to the various hydrogen ion concentrations for only one hour at room temperature. It will be seen from Table 1 that ectromelia haemagglutinin behaved in much the same way as vaccinia haemagglutinin. The complement fixing antigen was also very stable over a considerable range of hydrogen ion concentration. These results are rather similar to those obtained by van den Ende, Polson and Turner (1957) with the rabies virus soluble antigen, which they found to be fairly stable from pH 6·0 to 10·0. Treatment at pH 2·0 and 12·0 made the antigen anti-complementary, but reduced the titre by at least ¼ and ⅛ respectively. The LS antigen of vaccinia is stable at a pH at least as low as 4·5 (Craigie and Wishart, 1936b) and probably at a pH at least as high as 8·6 (Shedlovsky and Smadel, 1942) as measured by the precipitin titre.

The results obtained on the heat stability of the complement fixing antigen are rather similar to those of MacDonald and Downie (1950), although they found no more than a twofold reduction in titre when testing similar antigens against antivaccinial and anticowpox sera. A suspension of normal chorio-allantoic membrane put through with the first experiment gave a trace reaction at 1/5 which was abolished on heating, but when similar unheated suspensions have been spun for 5×10^6 g. minutes, the supernatant, even when undiluted, has shown no fixation in the presence of antivaccinial serum. It seems most likely that ectromelia soluble antigen, like the much studied vaccinia soluble antigen, possesses both a heat stable and a heat labile component and that Mayr, Herrlich and Mahnel are wrong in assuming that the heat labile complement-fixing antigen isolated from infected chorio-allantois is merely a normal tissue component.

The effect of heat on the haemagglutinin of the vaccinia has been studied by numerous workers and although the reported results have been rather variable the general consensus of opinion is that the haemagglutinin is remarkably heat stable (Chu, 1948; Tagaya, 1954a; Tagaya and Otani, 1954; Mayr, 1956). However, Gillen, Burr and Nagler (1950) separated what they considered to be two distinct haemagglutinins by high speed centrifugation (17,000 r.p.m. for three hours); one, which was associated with the virus, was heat stable, and the other, which remained in the supernatant, was heat labile. Their results were, in general, confirmed by Briody (1951) and Tagaya (1954b), who attempted to get rid of the majority of the virus elementary bodies by differential centrifugation before separating the haemagglutinin fractions, but there remained some

difference of opinion over whether more than one haemagglutinin was involved and the precise heat lability of the "soluble" haemagglutinin.

The results on ectromelia haemagglutinin, as far as they are comparable, are much the same as those obtained by the latter workers with vaccinia haemagglutinin. Also, when a vaccinia haemagglutinin was run in parallel with the ectromelia haemagglutinin in Experiment 1, both gave similar results. The total centrifugal force used in the other experiments was greater than that used by any of the above workers and no attempt was made to separate the virus elementary bodies from the heavier haemagglutinin. In similar preparations some haemagglutinin was still present in the supernatant, perhaps identical with the "labile" haemagglutinin found by Gillen *et al.* with vaccinia virus and described by them as producing "landslide" agglutination of the red cells. However, the ectromelia haemagglutinin showed little more than ± reactions in any tube and this "titre" was reduced only by one-half on heating at 56° C. for 30 minutes, although heating at 68-70° C. for 30 minutes did reduce it by 1/8.

It is difficult to be certain that the heat labile haemagglutinin is non-specific as Mayr (1956) claims, but it seems to react to immune serum as if it were specific. For instance, the original membrane suspension of Experiment 3, Table 3, that showed a titre of 1/480 when unheated, of 1/120 after heating at 56° C. and 1/60 after heating at 68-70° C., showed no agglutination from 1/2 when titrated in the presence of an undiluted antivaccinial rabbit serum (titre 1/480). Similarly, the washed deposit, which had a titre of 1/1280 before heating, of 1/960 after heating at 56° C. and 1/320 after heating at 68-70° C., had a titre of 1/2 under the same conditions. Both series reacted normally in the presence of serum from the pre-inoculation bleed.

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RADIATION INDUCED "ACCEPTANCE" AND THE IMMATURE HAEMOPOIETIC HOMOGRAFT

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SUMMARY.

Following lethal whole body irradiation and homologous embryonic haemopoietic grafting, transplantation of the matured grafted material was made into further lethally irradiated test animals with the object of demonstrating tolerance. This has been judged by the ability of such transplanted material to avoid secondary disease in a foreign host environment.

INTRODUCTION.

Jacobson *et al.* (1950) and Lorenz *et al.* (1951) demonstrated that by injections of splenic homogenates survival could be obtained in mice that had otherwise received a lethal dose of whole body irradiation. Progress in experimental protection against lethal total body irradiation has shown that successful resuscitation depends on the "take" of a haemopoietic graft. The cytological proof of the concept of radiation chimaerism dates only from the work of the Harwell group some three years ago (Ford *et al.*, 1956; Ford *et al.*, 1957). It was then shown that during the process of irradiation and bone marrow transplantation a change was effected in the irradiated host, abrogating the normal laws of tissue transplantation, allowing the foreign haemopoietic graft to colonise and persist indefinitely. In the following years the immunological tangles attending the close and unnatural approximation of two genetically dissimilar tissues has inspired much original thought. Avoidance of secondary radiation disease, a syndrome resulting from a chronic antigen-antibody reaction dependent on the degree of genetic divergence between the host and donated grafted tissue (Ilbery *et al.*, 1958; Uphoff and Law, 1959) is of prime importance. It is believed from serological (Grabar *et al.*, 1957) and immunological evidence that this underlying immunological reaction is in the direction of graft versus host. Using immunologically immature donor material, Uphoff (1958) and Barnes *et al.* (1958a) were able to avoid this reaction in some chimaeras in which the donation of genetically dissimilar mature tissue had previously proved inevitably fatal.

The question then arose as to whether the use of embryonic, foetal or new born tissue gives an improved chance of immunological paralysis of donor cells to the host's antigens taking place (Bauer *et al.*, 1956) or if there is adaptation

or tolerance on the part of the donor cells for host antigen. When adult haemopoietic tissue has been donated it has been postulated (Barnes *et al.*, 1958), in the case of the longer lived radiation chimaeras where these chimaeras have not developed secondary disease or where they have developed the disease and later recovered, that the graft is immunologically paralysed as regards the host.

Secondary radiation disease.

When the CBA strain is the irradiated host, donor haemopoietic material from CBA, C3H, T6/+, A, DBA and C57 gives much the same protection against the acute effects of lethal whole body irradiation. However, three weeks from the time of haemopoietic grafting, mice resuscitated with bone marrow other than of the same strain as the host begin to show varying patterns of survival according to the degree of genetic disparity at the H-2 locus (Gorer *et al.*, 1948). Some mice commence to exhibit a continuing loss of weight; they develop a hunched attitude and there is a failure of the coat to grey (early greying is seen some three weeks following irradiation in isologous chimaeras, e.g. CBA/CBA[1]). Most significant is the onset of diarrhoea which persists until either the mouse dies showing the fully developed secondary radiation syndrome or it commences to recover. No CBA mouse given CBA bone marrow in this laboratory has suffered this syndrome so that it is not a function of isografting. It is commonly seen in homografting and in its most blatant form in heterografting. Not all CBA mice treated with C3H marrow escape secondary disease, although they share the H-2^k locus. But they are not as much affected with the disease as those CBA treated with T6/+ or A which show an increasing incidence (Barnes *et al.*, 1957). CBA/DBA and CBA/C57 have given uniformly fatal results by 90 days post-irradiation and bone marrow replacement.

The *post mortem* appearance of mice showing this syndrome and the results of histological examination have been recorded by Ilbery *et al.* (1958), a notable feature being the atrophy of lymphoid elements.

Materials and Techniques.

Mice.

Mice of the following inbred strains CBA, C57BL, DBA/2 have been maintained by strict sib-mating and reselection of lines in this laboratory for two years. The annotation of the (DBA × C57) F_1 hybrids follows the convention of placing the doe first. Mice were subjected to irradiation when they had attained an age of three months.

The following doses, previously estimated as the L.D.99, were given:—

Whole Body Irradiation	
Strain	Rads
C57BL	900
DBA/2	950
CBA	1,050

[1] The convention of recipient/donor will be followed in description of chimaerism.

Irradiation.

The radiation source was a Theratron Co^{60} Unit made available by Royal Prince Alfred Hospital, Sydney. The initial dose rate calculated in December, 1957, was 43 rads/min. gamma irradiation at the 99 p.c. isodose. Dose rates ranged from forty to forty-three rads/min. allowing for the decay of radiocobalt over the period of the experiments. A 10 cm. diameter irradiation cage made of perspex allows up to seven three-months-old mice to be simultaneously irradiated. There is a 2 p.c. isodose fall off from the centre to the edge of the irradiation cage. The source was rotated through 180° half way through the treatment so that the mice were irradiated equally from above and below.

Haemopoietic donor tissue.

Bone marrow sufficient for the treatment of four, five or six mice was obtained from the shafts of two femurs of adult donors. The epiphyses were removed and the marrow washed from the cavity of the diaphysis by means of a current of physiological saline introduced through a 23 needle. The plugs of bone marrow were then dispersed by drawing up and down in a Mantoux syringe. Injection into the tail veins of mice was made with this syringe and a 26 needle. The age of the donors was three or four months and it was usual to use donor material of the same sex as the host.

Embryo spleen was used in preference to embryo liver, but at a foetal age of less than seventeen or eighteen days sufficient spleen could not be obtained and recourse was had to foetal liver. In either event the haemopoietic material was macerated in physiological saline by an M.R.C. type electrical mincer and, the coarse particles having been allowed to settle, administered as above.

EXPERIMENTAL.

The experimental results shown in Table 1 were designed to illustrate the leading points already made and to serve as a base for the following bone marrow transplantation experiment and the resulting discussion.

Establishment of the phenomenon of secondary radiation disease and the direction of the immune reaction as graft against host.

Twenty C57BL, twenty-two CBA and twenty-three DBA mice received lethal whole body irradiation. Mice given no further treatment were all dead by the thirteenth day. The remaining twelve C57BL mice were divided into three equal groups. Within sixteen hours of irradiation the first group received 2×10^6 cells each of adult DBA bone marrow, the second group 2×10^6 cells each of adult (DBA × C57) F_1 hybrid bone marrow and the third embryonic material by intravenous injection of the tail veins. The remaining thirteen CBA mice were divided into groups of six and seven, the former receiving $2{\cdot}2 \times 10^6$ cells each of adult C57BL bone marrow and the latter embryonic material, intravenously within forty hours of irradiation. The remaining thirteen DBA were divided into two groups of five and eight respectively, the former receiving $2{\cdot}6 \times 10^6$ cells each of adult (BDA × C57BL) F_1 hybrid bone marrow and the latter embryonic material, intravenously within sixteen hours of irradiation. The results show, in the C57BL/DBA and CBA/C57BL chimaerical combinations

where adult haemopoietic material has been donated, the invariable occurrence of secondary radiation disease within the fourth week followed by death within the next few weeks. It is seen clearly, however, that when the F_1 hybrid is the source of adult donor bone marrow to either parental strain, C57BL or DBA, with the exception of one death due to the acute radiation syndrome all chimaeras survived longer than ninety days without the occurrence of secondary radiation disease. This is the situation where the graft is incompatible with the host so that if the host were responsible for the immunological reaction it certainly would have been apparent here.

Amelioration of secondary radiation disease by the use of immunologically "null" material.

Four C57BL mice received 750,000 cells each of embryo (C. 17 days) DBA spleen intravenously following lethal whole body irradiation. Seven CBA and eight DBA mice similarly lethally irradiated received intravenous injections of embryonic liver, the former 5×10^6 cells each of C57BL within forty hours of irradiation and the latter 8×10^6 cells each of C57BL within sixteen hours of irradiation. The use of embryonic liver or spleen considerably modified the outcome in these incompatible chimaerical combinations as shown when adult tissue was used. Of the thirteen chimaeras that survived the acute effects of radiation, i.e. lived longer than twenty-one days, ten survived longer than ninety days. However, secondary radiation disease was apparent in varying degrees in five of the thirteen survivors. Of the CBA/C57BL chimaeras one died on the thirtieth and one on the fortieth day, both ungreyed and exhibiting diarrhoea, and two others did not commence to grey until the tenth week post-irradiation. Of the DBA/C57BL chimaeras one died on the twenty-seventh day, early in the secondary disease period, having failed to regain weight and another failed to grey and commenced to have diarrhoea late in the second month, dying one hundred and two days post-irradiation (Table 1).

Although it can be seen that over half the mice survived ninety days post-irradiation when resuscitated with embryonic tissue as compared with the invariably fatal outcome by the end of two months when adult tissue was the donor material, nevertheless secondary disease was not entirely eliminated, even if only seen in a mild form. By the use of immature donor material, is there a tendency for the graft to tolerate or adapt itself to the host antigen rather than to develop immunity against the host antigen as is shown by the onset of secondary disease when adult tissue is donated? Or rather, is it the very immaturity of the donor tissue which facilitates immunological paralysis of graft against host? As embryonic tissue will have matured during ninety days' sojourn in the radiation chimaera, retransplantation of this tissue should resolve this question.

TABLE 1.

Pattern of secondary disease following irradiation and haemopoietic grafting.

Host	Days following irradiation (5 | 10 | 20 | 30 | 40 | 50 | 90)	Donor	Treatment	
C57BL	XX XX XXX X			
	Xs XsXs Xs	DBA	Adult	bone marrow
	O O O O	DBA/C57	Adult	bone marrow
	X X O O	DBA	Embryo	spleen
CBA	X XX XX XXXX			
	X X Xs sXXs Xs	C57BL	Adult	bone marrow
	O O O X Xs Xs O	C57BL	Embryo	liver
DBA	X XX XX XXXXX			
	O O O X O	DBA/C57	Adult	bone marrow
	O O O X X X Xs O	C57BL	Embryo	liver

X=dead, Xs=dead from secondary disease, O=alive.

Tests for secondary radiation disease on retransplantation.

TABLE 2.

Transplantation of DBA/C57BL chimaerical haemopoietic material into lethally irradiated DBA and C57BL mice.

	950 rads	Survival at 90 days	900 rads	Survival at 90 days
Chimaera 1	2 DBA	1/2*	3C57BL	3/3
Chimaera 2	3 DBA	3/3	2C57BL	2/2
Total		4/5		5/5

*1 dead at 64 days, greyed and not exhibiting diarrhoea.

Two of the DBA/C57BL chimaeras resuscitated with embryonic liver were sacrificed six months after lethal whole body irradiation. The donor C57BL haemopoietic and lymphopoietic tissue must be considered well matured after this length of time. The donors were well greyed healthy looking mice that had at no time showed any form of secondary disease. No abnormality was seen in the macroscopic or microscopic examination of their bone marrow, spleens or lymph glands. 2×10^6 bone marrow cells per mouse from these chimaeras were injected intravenously into lethally irradiated DBA and C57BL mice. Some mice had commenced to grey by the fourth week and all were grey by the sixth week post-irradiation and replacement with chimaerical bone marrow. Diarrhoea was not observed. Apart from one greyed DBA mouse that died about the sixty-fourth day and had undergone *post mortem* degeneration, all mice survived ninety days. One DBA and one C57BL killed at the ninety-ninth day were well greyed, healthy mice, their lymphoid tissue was fully developed and no abnormality was seen in the myeloid or lymphoid elements by microscopic examination.

Test for maturity of embryonic donor tissue and for persistence of the phenomenon by retransplantation.

Seven CBA mice received 1050 rads of Co^{60} gamma irradiation and were resuscitated with 5×10^6 embryonic (late third week) cells from the C57BL strain. Four survived to three months and the haemopoietic material from these healthy well greyed mice was transferred to DBA, CBA and C57BL lethally irradiated recipients. Bone marrow was used at a dosage of 2×10^6 cells to each mouse.

All C57BL recipients survived, whereas all DBA died. An interesting observation as regards the CBA given CBA/C57BL chimaerical bone marrow was that the violent reaction, manifested by diarrhoea and failure of the coat to grey always shown when adult tissues are used, was considerably reduced in the four mice that did die following administration of this adult chimaerical tissue. Although all were greatly wasted, two had commenced to grey and

TABLE 3.

Survivors to three months of lethally irradiated mice given CBA/C57BL chimaerical haemopoietic tissue.

	DBA	C57BL	CBA
# 218	—	2/2	2/3
# 224	0/2	2/2	1/2
# 225	0/2	2/2	1/2
# 226	0/2	2/2	1/2
Total	0/6	8/8	5/9

only one showed mild diarrhoea. It appeared that these mice were partially tolerant, the partially tolerant-partially immune situation (Barnes and Loutit, 1959). However, the results of further transplantation of the initial chimaerical bone marrow through CBA and C57BL to further CBA and C57BL will be interesting especially in the case of the transfer through C57BL where the chimaerical marrow has been out of contact with CBA antigen.

DISCUSSION.

Although recovery from the lethal effects of whole body radiation can be satisfactorily ameliorated (sterility still supervenes and there are still the radiation ageing effects such as diminution of life span and formation of cataracts) by the intravenous injection of isologous bone marrow, the use of homologous material is accompanied by varying degrees of secondary radiation disease. In that the use of F_1 hybrid bone marrow does not register secondary disease it is clear that the direction of the immunological reaction is graft versus host. Whereas C57BL/DBA, H-2^b/H-2^d and CBA/C57BL, H-2^k/H-2^b chimaerical combinations together with those reported by Uphoff and Law (1959) which included DBA/C57BL, H-2^d/H-2^b present an invariably fatal immunological reaction when the donor tissue is of adult origin, the outcome is in contrast when the donor material is in the immunologically "null" period.

The Harwell group by means of donations of chromosomally marked haemopoietic cells (T6/+), intravenously injected into lethally irradiated hosts (CBA), have demonstrated that all the dividing cells in the recovering bone marrow, lymph nodes, spleen and thymus were of donor type. Since these organs play a vital rôle in the immune responses their destruction by radiation and replacement by cells of the donor animal have far-reaching immunological consequences. In unpublished observations this group had retransplanted the marked donor tissue from surviving chimaeras (CBA/T6/+) to lethally irradiated mice of the original host strain (CBA). The chimaeras resulting from the retransplanted myeloid elements were again not free from secondary disease. The mechanism

in these circumstances would appear to be that the T6/+ graft had established an immunological balance with the individual CBA, not to the CBA strain as a whole; so that on retransplantation to CBA hosts it had again to make this balance, sometimes, as in the original transplantation, secondary disease being the unfavourable result. Reviewing the evidence at that time we (Barnes *et al.*, 1957) felt that survival of chimaeras in the homologous situation was due to an immunological paralysis (Felton, 1949) of the graft for the tissues of the host.

Can the avoidance of secondary disease using immature embryonic tissues be ascribed also to immunological paralysis "the immaturity of the tissue facilitating this mechanism" (Bauer *et al.*, 1956)? Embryonic myeloid and lymphoid elements do mature in their new environment as shown by Ilbery (1958) in the case of mouse/rat chimaeras by the onset of secondary disease on the average some three weeks later in those chimaeras resuscitated with foetal as opposed to those resuscitated with adult haemopoietic tissue. In the more favourable homologous situation it is conceivable that the more slowly maturing tissue could avoid secondary disease attributable to this mechanism. Recalling that although T6/+ grafted tissue could adapt itself (whether immunological paralysis is the mechanism or not) to live symbiotically in one irradiated CBA and that there was no guarantee that it could repeat the performance when transplanted to another CBA, it was decided to test the hypothesis of immunological paralysis in the case of the DBA/C57BL chimaeras surviving by virtue of the use of embryonic splenic tissue. It was to have been expected that the C57BL bone marrow having matured during its sojourn within the DBA, would now, as adult tissue, on retransplantation to DBA, result in the invariably lethal outcome characteristic of the incompatibility of these two strains when adult tissue is used. As can be seen from Table 2, however, there was a clear-cut "take" of C57BL bone marrow in the lethally irradiated DBA. Thus it seems that there has been adaptation of the C57BL cells for DBA mice.

Before accepting adaptation as an explanation for this phenomenon it is necessary to exclude the possibility that reversion may have taken place or that the C57BL cells were still immature. However, five out of five lethally irradiated C57BL mice greyed normally and did not exhibit diarrhoea. If, as has been believed, reversion takes place in homologous chimaerism, then one would have expected the bone marrow, having reverted from donor type C57BL to host type DBA, to cause a fatal secondary disease on transplantation to lethally irradiated C57BL hosts. Although reversion is seen (Ford *et al.*, 1957) in the case of mouse/rat radiation chimaerism, from cytological evidence it is apparent that all the reverting mouse cells have arisen from only a few radiation damaged cells. The mouse may be living by virtue of a few clones of cells. It was argued in that paper that whereas rat cells were not acceptable in the irradiated mouse environment, homologous cells were more acceptable than badly damaged host type cells. Whatever is the mechanism for this "physiological competence"

it would also seem to have applied in the case of these transplantation tests where the unirradiated C57BL cells, having adapted themselves to the DBA environment, are physiologically more competent to ensure the survival of lethally irradiated DBA mouse than any of its own remaining radiation damaged cells. Another possibility remains and will be looked for cytologically at a later date. This is that there may have been introduced into the lethally irradiated DBA resuscitated with DBA/C57 chimaerical bone marrow a mixed cell population of C57BL cells and a few radiation damaged DBA cells capable of reversion. If this were the case, the C57BL cells might act as a blood forming transfusion until a clone or clones of radiation damaged DBA cells (bearing chromosome aberrations) could establish a blood forming graft. If the bone marrow in the case of the CBA/C57BL radiation chimaeras, the result of a graft of embryonic tissue, were still immature it would on retransplantation to lethally irradiated DBA as well as to CBA and C57BL result in avoidance of secondary disease and this would explain the result of the DBA/C57BL transplantation test. That death from secondary disease resulted in the DBA mice ($H\text{-}2^d/H\text{-}2^b$) is evidence that maturity of donor tissue is obtained and the survival of 5/9 CBA further evidence that there is adaptation of donor tissue to host.

The question as to whether the host's immunological mechanism has been destroyed, is permanently neutral or is in a state of actively acquired tolerance to donor type cells in the sense of Billingham, Brent and Medawar (1955) has been considered elsewhere (Barnes *et al.*, 1958b). At that time we felt that the host had an actively acquired tolerance for the graft. But Billingham claims that actively acquired tolerance represents the effect of a complete antigen on an immature subject. Whilst this may apply to the immature grafted haemopoietic material in relation to the lethally irradiated host's antigen, where avoidance of secondary disease is obtained, it is not felt that tolerance in this sense applies to the host. Radiation "tolerance" was suggested by Ilbery (1958). However, this implies some activity on the part of the host and a term such as "radiation induced acceptance" might more deservedly describe the passive nature of the host's immune mechanism. It is probably not necessary in a physiologically competent situation, i.e. homologous chimaerism where all dividing cells of bone marrow and lymph glands are of donor type as opposed to the reverting state in heterologous chimaerism, to invoke an active immunological mechanism to explain the non-reactiveness of the host towards the graft.

In conclusion, it is postulated that it is the graft cells predominating and controlling the immune response which, in the case of the situation where adult tissue has been used to effect chimaerism, exhibit immunological paralysis, and in the case where immunologically null tissue has been used exhibit actively acquired tolerance for the host.

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THE DYNAMIC RED CELL STORAGE FUNCTION OF THE SPLEEN IN SHEEP

II. JUGULAR HAEMATOCRIT FALL AFTER SOME TRANQUILLIZING AGENTS, PARTICULARLY CHLORPROMAZINE

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SUMMARY.

Chlorpromazine (0·46-0·7 mg./kg. i.v.) caused profound fall of haematocrit in sheep. Its virtual abolition by splenectomy and its inverse relationship to splenic gamma radiation surface counts in intact sheep prepared with ^{51}Cr-labelled red cells showed this to be due almost entirely to splenic relaxation and consequent increase of red cells held there. Plasma dilution through water shift played only a small part in the haematocrit fall Similar haematocrit fall, and its virtual abolition by splenectomy, occurred after dihydroergotamine but not after Dibenamine. Both these agents, as well as chlorpromazine, were shown to reduce the haematocrit response to adrenaline or *nor*-adrenaline. Haematocrit fall also occurred after pentobarbitonum sodium at a dose sufficient to produce obvious central effects. The relative importance of ataraxis and of splenic adrenergic blocking in the haematocrit-reducing effect of chlorpromazine in sheep could not be wholly resolved, but the central effects are probably more important. Haematocrit fall, abolished by splenectomy, was also observed after mepazine (2·3 mg./kg. i.m.). The effect after meprobamate (9·4 mg./kg.) was slight.

INTRODUCTION.

It has been shown that the jugular haematocrit of a sheep may fluctuate greatly, depending upon whether the animal is excited or placid when the samples are collected; in extreme instances, values during excitement were almost twice as great as when the sheep were gently restrained and soothed in quiet surroundings for about 15 min. by a sympathetic attendant, a procedure referred to as "holding" (Turner and Hodgetts, 1959). These changes were shown to be inversely related to the degree of contraction or relaxation, i.e. emptying or filling of the spleen, and to the proportion of the total red cell mass retained therein in dynamic equilibrium with the circulating blood. In view of the increased retention of red cells in the spleen and the consequent lowering of jugular haematocrit as a result of what may be referred to as psychological tranquillization, it was expected that haematocrit fall might also occur under

the influence of pharmacological tranquillization by ataractic drugs. In this communication experiments are described which show that this is so, and an attempt is made to throw some light on the mechanism of the effects observed.

MATERIALS AND METHODS.

The general methods of investigation, and the techniques used, are as described in the earlier publication. All haematocrit values are corrected for trapped plasma, and where indicated are further adjusted to annul the effect of water shifts, by calculating what the haematocrit value would have been if the plasma protein concentration (PP), and by inference the plasma volume, had remained constant during the relevant period. It is assumed that such water shifts did not appreciably alter the packing volume of the red cells.

Chlorpromazine hydrochloride, used as Largactil (May and Baker) is 3-chloro-10(3′-dimethylamine-*n*-propyl)-phenothiazine; it was administered intravenously (i.v.) in a dose of 25 mg. in 10 ml. normal saline, or intramuscularly (i.m.) in twice this amount. Mepazine, used as Pacatal (Warner & Co.) is 10-(N-methyl-3-piperidylmethyl)-phenothiazine; 4 tablets (= 100 mg.) were ground up in 5 p.c. glucose solution, and injected i.m. Meprobamate, used as Miltown (Lederle) is 2-methyl-2-*n*-propyl-1,3-propanediol dicarbamate; one tablet (= 400 mg.) was ground up in 4 ml. saline and injected i.m. Dibenamine is N-(2-chloroethyl) dibenzylamine hydrochloride; 800 mg., dissolved in 80-100 normal saline, sometimes after first taking up in 8 ml. propylene glycol, were injected i.v. Dihydroergotamine, used as the methane sulphonate, was used as a 0·2 p.c. solution (DHE-45, Sandoz Products), and administered i.v.

RESULTS.

A. *Haematocrit fall after some tranquillizing agents.*

Effect of chlorpromazine upon jugular haematocrit. Five intact sheep were investigated. In the doses used (0·7-0·48 mg./kg.) chlorpromazine produced little obvious effect upon the sheep, except a lack of interest in their surroundings, slight somnolence and a tendency to crouch a little when handled; they remained completely conscious.

Sheep 502 received 0·7 mg./kg. i.v. Before the injection, haematocrit was 40·6. After injection the sheep was released, and was retaken at intervals for repeated blood sampling by venipuncture. Haematocrit fell in a manner resembling that during barbiturate anaesthesia, but somewhat less quickly, reaching 31·2 after 15 min. and 28·8 after 60 min. (29 p.c. decrease from the initial level). Evidence of slight expansion of plasma volume through water shift was observed during the first 60 min., PP decreasing from 7·74 to 7·33 gm./100 ml., but thereafter PP gradually returned to the initial level and then rose beyond it to 7·9 gm./100 ml. Adjustment was made as described to annul the effect upon red cell concentration of the changes in plasma volume suggested by the changes in PP. Minimum "adjusted" haematocrit was calculated as 29·6, representing 27 p.c. decrease in the relative number of red cells in the jugular blood from the initial level. Haematocrit then began to rise slowly and by the end of the fourth hour had reached 36·7 (Fig. 1).

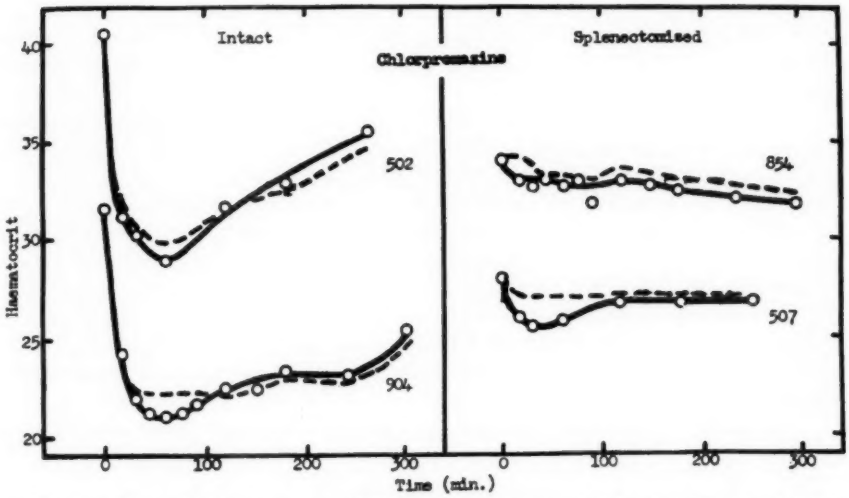

Fig. 1. Effect of chlorpromazine (0·7 mg./kg. i.v.) upon jugular haematocrit of intact and of splenectomized sheep. The broken lines are drawn through the haematocrit values after adjustment to annul the effect of water shifts upon plasma volume and red cell concentration.

Next morning haematocrit was 38·5. Chlorpromazine was again injected, the dose on this occasion being doubled (1·4 mg./kg.), and administered i.m. The sequence of events was very similar to that following i.v. injection, although haematocrit fell a little less quickly at first. The minimum was again reached after 60 min., when 30 p.c. decrease from the initial value was recorded; again evidence of slight water shifts was observed, and after appropriate adjustment the minimum "adjusted" haematocrit was calculated as 26·6 (29 p.c. decrease). Haematocrit then gradually returned to normal.

Sheep 904 received 0·7 mg./kg. i.v. After injection, haematocrit fell from 31·6 to 21·0 (34 p.c. decrease from the initial value) after 60 min. There was again slight plasma dilution during the first 60 min. and over-compensatory recovery later. When appropriate adjustments were made, the relative numbers of red cells in the jugular blood were found to have decreased 29 p.c. after 60 min. (Fig. 1).

Sheep 910 and 903 received 0·63 and 0·48 mg./kg. i.v. respectively. This procedure was repeated on a number of occasions within the period of a few days, as part of the experiment to determine the inhibitory effect of chlorpromazine pre-treatment, 30 min. previously, upon haematocrit rise after injection of sympathomimetic amines. The mean of the initial values, which differed only a few p.c. on the various occasions, was 33·9 for each sheep, and this mean value was used in the calculations of p.c. haematocrit fall after chlorpromazine.

The decreases after 30 min. (probably not at their minima, which usually occur after about 60 min.) were 22 and 24 p.c. respectively of the initial values. Because of the use of mean values, adjustment for the effects of water shifts was not made.

Sheep 895 received 0·56 mg./kg. i.v. In this instance haematocrit reached minimum values about 45 min. after injection, falling from initial value 30·0 to 23·5, or after adjustment for water shift, 24·5 (18 p.c. decrease).

The main data of these experiments are summarized in Table 1.

Effect of mepazine upon jugular haematocrit. Injected i.m. into sheep 822, mepazine (2·3 mg./kg. i.m.) caused haematocrit fall from initial value 35·3 to minimum value 29·0 (18 p.c. decrease) about 100 min. after injection, followed by slow return towards the initial levels (Fig. 2). The departures from the smooth curve, particularly that at 110 min., were associated with some struggling and resistance to bleeding, a phenomenon which did not occur with chlorpromazine.

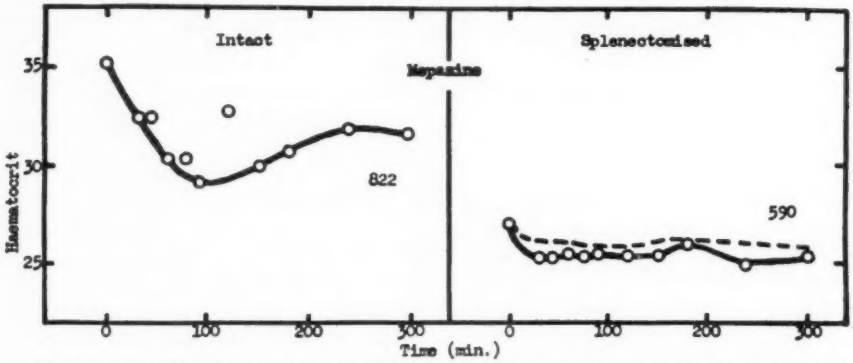

Fig. 2. Effect of mepazine (2·3 mg./kg. i.m.) upon jugular haematocrit of intact and of splenectomized sheep. The broken line is drawn through the haematocrit values of splenectomized sheep 590 after adjustment for the effect of water shifts; these did not occur in intact sheep 822.

Water shift was not evident until near the sixth hour, when slight plasma dilution occurred. On a subsequent occasion this sheep reacted in a similar fashion to the same dose of mepazine, haematocrit falling from initial value 35·3 to minimum value 29·7 (16 p.c. decrease) after 90 min.

Effect of meprobamate upon jugular haematocrit. Injected into sheep 879, meprobamate (9·4 mg./kg. i.m.) caused only a small haematocrit fall, from initial value 31·6 to 29·0 (8 p.c. decrease) at 90-120 min. after injection. This was not accompanied by evidence of water shifts. Having reached a minimum, haematocrit then slowly began to rise, but was still 6 p.c. below the initial level when observations ceased 6¾ hr. after injection. Further experiments were not made.

TABLE 1.

Haematocrit fall after chlorpromazine.

Sheep	Chlorpromazine (mg./kg.)	Route	Before injection		After injection			
			Haematocrit	PP (g./100 ml.)	Minimum haematocrit (observed)	PP (g./100 ml.)	Minimum haematocrit (adjusted for water shift)	Decrease† (p.c.)
Intact								
502	1·4	i.m.	38·5	7·74	26·9	7·56	27·4	30 (29)
502	0·7	i.v.	40·6	7·74	28·8	7·38	29·6	29 (27)
904	0·7	i.v.	31·6	6·66	21·0	6·12	22·5	34 (29)
910	0·63	i.v.	33·9	—	25·9*	—	—	— (24)
895	0·56	i.v.	30·0	6·48	23·5	6·12	24·5	22 (18)
903	0·48	i.v.	33·9	—	26·4*	—	—	— (22)
Splenectomized								
507	0·69	i.v.	27·9	7·02	25·6	6·48	27·2	8 (3)
854	0·56	i.v.	34·2	6·66	31·7	6·30	32·9	7 (4)

* These were determined 30 min. after injection and are therefore probably somewhat in excess of the minimum values.

† The first value is calculated on the haematocrit as observed, the value in parenthesis on the haematocrit after adjustment for water shift.

B. *Relationship of haematocrit fall to the spleen.*

Chlorpromazine: Evidence of splenic filling as revealed by ^{51}Cr-marked red cells. Sheep 895 had been prepared on the previous day by i.v. injection of ^{51}Cr-marked red cells. An intra-jugular polythene cannula was established. Haematocrit and splenic surface counts were determined. After injection of chlorpromazine i.v., counting was continued over the splenic region, and blood samples were collected *per cannulam* for haematocrit determination at intervals. During the subsequent 45 min. haematocrit fell from 30·0 to 24·5 (18 p.c. decrease), while surface gamma radiation counts increased from 5850/min. to 6950/min. (19 p.c. increase), indicating a complementary increase of red cells in the spleen (Fig. 3). The virtual equivalence of the p.c. haematocrit decrease and p.c. count increase is probably fortuitous. Thereafter, haematocrit began to rise slowly, and surface counts to decrease.

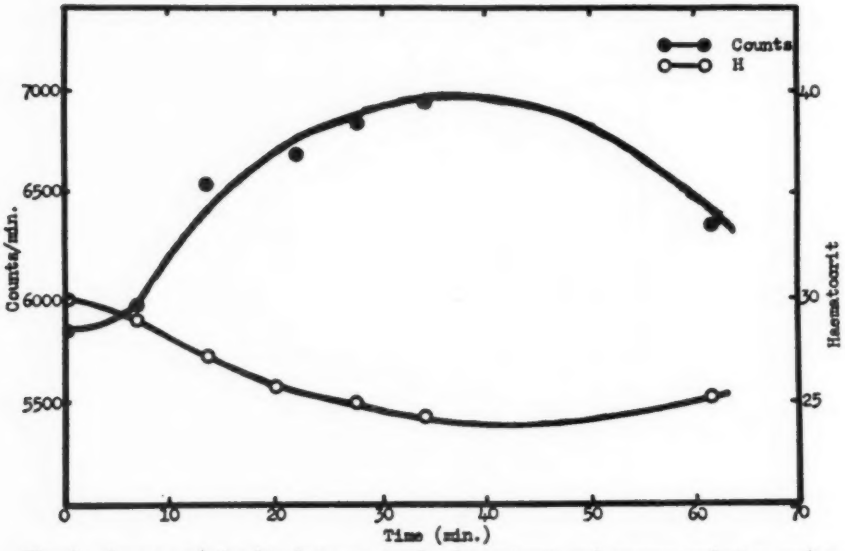

Fig. 3. Inverse relationship between jugular haematocrit and gamma radiation surface counts over the splenic region of sheep 895, previously prepared with ^{51}Cr-labelled red cells and injected at time 0 with chlorpromazine (0·56 mg./kg. i.v.).

Chlorpromazine: Effect of splenectomy upon haematocrit fall. Two splenectomized sheep, 507 and 854, Fig. 1, were injected i.v. (0·7 mg./kg.). There were slight falls of haematocrit, which decreased within 30 min. from 27·9 to 25·6 in sheep 507, and from 34·2 to 33·8 in sheep 854. In both sheep, decreased plasma specific gravity (PSG) indicated slight plasma dilution due to water shifts, and when appropriate adjustments were made the minimum adjusted values were

calculated as 27·0 and 32·4 respectively (3 p.c. and 5 p.c. decrease in numbers of red cells in jugular blood).

Mepazine: Effect of splenectomy upon haematocrit fall. Splenectomized sheep 590 received mepazine i.m. (2 mg./kg.). Haematocrit fell only slightly from initial value 27·0 to values varying from 25 to 26 during the subsequent 5 hr. There was evidence of slight plasma dilution through water shift. Fig. 2 shows that, after a very slight fall in the relative numbers of circulating red cells, very little further change occurred.

Mechanism of increased splenic filling after chlorpromazine.

In considering this, the degree of splenic filling in the conscious sheep was assumed to be a function of the tonus of splenic musculature and possibly of vascular sphincters (Green and Kepchar, 1959), depending upon the flux of post-ganglionic sympathetic impulses along the splenic nerve, and increased filling was assumed to be a passive phenomenon, following lowering of tonus. Two hypotheses to explain increased splenic filling were considered, (a) chlorpromazine diminishes the flux of impulses, originating in the cerebral cortex, to the ganglia, as a result of ataractic action (i.e. emotional indifference to otherwise alarming stimuli normally present in the environment) or of cortical depression associated with somnolence, and (b) chlorpromazine in some manner blocks post-ganglionic impulses from exerting their effect upon splenic tonus. Some experiments which might help in assessing the relative importance of these two possibilities were therefore carried out.

Inhibition by chlorpromazine of haematocrit rise after sympathomimetic amines. Courvoisier *et al.* (1953) have demonstrated certain anti-*nor*-adrenaline and anti-adrenaline effects of chlorpromazine in dogs, e.g. antagonism to hypertensive effects and protection against otherwise fatal injections; but it was desirable to know whether it is able to inhibit haematocrit rise after these sympathomimetic amines in sheep. For this purpose the heights of the haematocrit responses following each amine, at two dose levels (2 and 10 μg./kg.) were determined in two sheep 910 and 903, both with and without prior preparation with chlorpromazine (0·63 and 0·48 mg./kg. i.v. respectively, 30 min. earlier). As a result of chlorpromazine injection, haematocrits fell from 33.9 to 25·9 and 26.4, respectively, in these two sheep, as already shown in Table 1. The results (Table 2) demonstrate that chlorpromazine does indeed strongly inhibit splenic contraction by these amines, the inhibitory effect being particularly striking against the small dose rate (2 μg./kg.).

Effect of Dibenamine upon haematocrit. If adrenergic blockade in the spleen is responsible for the haematocrit fall after chlorpromazine, this might also be expected after the powerful adrenergic-blocking agent Dibenamine. At the dose levels used by us (20 mg./kg.) it caused trembling and excitement which passed off within half an hour. Observations were made after 2-5 hr. In sheep 560, haematocrit fell from 34·9 initially to 31.2, but plasma dilution had

TABLE 2.

Haematocrit responses to nor-*adrenaline and adrenaline before and after injection of chlorpromazine.*

Sheep	Chlorpromazine (mg./kg.)	Haematocrit after			
		nor-Adrenaline		Adrenaline	
		2 μg./kg.	10 μg./kg.	2 μg./kg.	10 μg./kg.
903	—	33·2	35·6	37·4	36·5
903	0·48	27·3	31·6	28·9	33·5
910	—	30·3	35·9	36·2	37·0
910	0·63	25·4	30·3	25·6	31·0

occurred through water shift (PP fell from 6·84 to 6·12 gm./100 ml.). After adjustment to annul the effect of this, haematocrit was calculated as 33·7 (3 p.c. decrease), indicating only slight decrease in circulating red cells.

Inhibition of haematocrit rise after sympathomimetic amines and after excitement. Dibenamine had been shown by Nickerson and Goodman (1947) to prevent the haematocrit rise induced by fright and struggle in cats. Demonstration of anti-adrenergic effect upon the spleen of sheep was considered desirable.

In sheep 560 before treatment with Dibenamine, i.v. injection of adrenaline (36 μg./kg.) was followed by haematocrit rise to 38·4; after treatment with Dibenamine, adrenaline caused haematocrit to rise to 35·4. This represents a rather small inhibiting effect. In the next experiment, the dose level of amine (in this instance, *nor*-adrenaline) was therefore reduced.

In sheep 885, before Dibenamine, i.v. injection of *nor*-adrenaline (12 μg./kg.) was followed by rise of haematocrit to 36·4; after Dibenamine, the same dose of *nor*-adrenaline caused haematocrit to rise to only 29.4. In another experiment with this sheep, before administration of Dibenamine haematocrit rose to 34·1 when the animal was chased for 1 min.; after Dibenamine haematocrit rose to 33·2 when the animal was chased for 1 min. Less striking inhibition to the effects of excitement than to injected amines illustrates the generalization of Nickerson (1959) that adrenergic blocking agents inhibit responses to circulating sympathomimetics more readily than those to adrenergic activity.

Effect of dihydroergotamine upon haematocrit. In view of the virtual absence of haematocrit fall after Dibenamine, the effect of another adrenergic-blocking agent, dihydroergotamine (DHE), was examined. In the first series, increasing doses of DHE were injected i.v. into sheep 191. These necessarily had to be carried out over a period of some days. Slight differences of PSG in the initial samples occurred on the various occasions, and therefore to make the haematocrits in the series of experiments (Table 3) more comparable they have been adjusted with reference to the PP value of the first sample. In con-

TABLE 3.

Effect of dihydroergotamine (DHE) upon haematocrit, and upon haematocrit response after adrenaline (39 μg./kg. i.v.).

Dihydroergotamine μg./kg.	Effect upon haematocrit			Effect upon haematocrit response after adrenaline		
					Increase (p.c.)	
	Before	After	Decrease (p.c.)	Haematocrit	From initial value	From post-DHE value
Intact sheep 191						
14·0	32·8	25·9	21	37·3	14	44
42·0	35·3	27·4	22	36·6	4	34
168·0	34·3	28·1	18	35·6	4	27
1680·0	33·6	29·1	13	31·9	− 5	10
Splenectomized sheep 620						
1680·0	31·6	30·7	3	31·0	—	1

tradistinction to Dibenamine, DHE caused profound haematocrit fall (Table 3) within 15-30 min. The effect seemed to be maximal at about 14-42 μg./kg. (21-22 p.c. decrease), and progressively less, although still large, as the dose rate increased, and this was virtually abolished by splenectomy: in splenectomized sheep 620, there was only a trivial fall of haematocrit, namely, from initial 31·6 to 30·7, after DHE (1680 μg./kg.).

To determine the effectiveness of DHE as an adrenergic-blocking agent in sheep, treated sheep were then given adrenaline i.v. The dose chosen (39 μg./kg.) was probably too large to reveal this to the best advantage, but opportunity for further experiments did not occur. The increasing inhibition of haematocrit rise after adrenaline, as the dose rate of DHE increases, is shown in Table 3. The rise after adrenaline was completely inhibited by DHE at the rate of 1680 μg./kg. The inhibiting effect of DHE is even more striking when related to the post-DHE haematocrit values.

Experiments were also carried out to determine the effect of DHE (160 μg./kg.) upon haematocrit response to sensory stimulation. An in-dwelling polythene catheter was established in the jugular vein of sheep 904 isolated in a quiet room, and was led to the exterior. Before administration of DHE this sheep had responded strongly to entry of an observer into the room (24 p.c. increase). Two hours after treatment, haematocrit had decreased from the already low value (25·0) characteristic of the secluded sheep to the still lower value 22·0, i.e. 12 p.c. decrease. Entry of the observer now caused no haematocrit rise, although the sheep was aware of his entry, and, in fact, arose, and showed some resentment to venipuncture.

Effect of sedation with pentobarbitonum upon haematocrit. There was thus a sharp difference between the effects of Dibenamine and DHE upon haematocrit, the one causing virtually no effect and the other a profound fall. Since DHE is known to cause central depression whereas Dibenamine does not, and since chlorpromazine itself causes a small degree of somnolence, the effect of sedation by a barbiturate upon haematocrit was examined.

Sheep 786 and 799 were injected i.v. with pentobarbitonum sodium (7 mg./kg.), i.e. one-fourth of the average anaesthetic dose. In both sheep, haematocrits did not fall significantly after injection. These sheep remained fully conscious and appeared to be quite alert. Sheep 904 was injected with 10 mg./kg., i.e. about one-third of an anaesthetic dose. Before injection its haematocrit was 33·8. Immediately after injection it lost control of its legs, was unable to stand, and although apparently fully conscious appeared somewhat stupefied. Fifteen minutes later the animal was alert enough to attempt unsuccessfully to rise when attendants entered, but haematocrit had fallen 10 p.c. from the initial value. Thirty minutes after injection it was able to stand, but tended to stagger. At 45 minutes it was standing comfortably, without swaying, and resented handling and venipuncture; its haematocrit had now fallen slightly to 12 p.c. from the initial value. Haematocrit, which had slowly fallen continuously since injection, eventually reached a minimum, when adjusted for slight water shift, of 29·6 (12 p.c. decrease) after 90 min., with some irregular ephemeral rises associated with resentment to handling. At 255 minutes after injection, when observation ceased, haematocrit had risen to 31·3, i.e. to within 7 p.c. of the initial value. Thus pentobarbitonum sodium, which even at full anaesthetic doses gave no evidence of inhibiting appreciably the spleen-contracting effect of sympathomimetic catechol amines (Turner and Hodgetts, 1959), caused in the conscious sheep, when administered at a dose sufficient to produce signs of slight stupor and uncoordinated gait, a definite haematocrit fall, although differing somewhat from that after chlorpromazine by slower evolution and lower intensity.

DISCUSSION.

The remarkable haematocrit fall in sheep after chlorpromazine, shown to be mainly due to increased retention of red cells in the spleen, and only in a minor degree to plasma dilution through water shift, recalls a report by Hoe and Wilkinson (1957) that in dogs haematocrit fell by a variable amount (4-42 p.c., mean 23 p.c.) 90 min. after i.m. injection of chlorpromazine (0·9 mg./kg.). They referred to this phenomenon as haemodilution, adding that "no attempt was made to discover the cause of the dilution or the nature of the diluent". Martin and Beck (1956) recorded profound decrease (37-52 p.c.) in jugular red cell count of horses 24-48 hours after four daily doses of chlorpromazine (1-2 mg./kg. i.m.); the count did not return to normal in one horse until 13 days later. The mechanism was not determined, but they speculate on the possi-

bility of haemolysis. We occasionally observed faint haemoglobin tinting of plasma in sheep 24 hours after chlorpromazine, but do not consider this played any significant part in the transient haematocrit fall described here. The only other reference to the effect of chlorpromazine upon peripheral red cell concentration known to us is by Le Blanc (1958), who recorded a very small (4 p.c., and hence probably insignificant) decrease of red cell count in rats given chlorpromazine (10 mg./kg., intraperitoneally). However, the first post-chlorpromazine cell counts were recorded 4 hours after injection, a period which in sheep is well past the optimum period (60-90 min.) for observing maximum effects; furthermore, the importance of splenic control of peripheral red cell concentration is not well defined in this species. In view of the similar importance of the spleen in dogs and sheep as a regulator of peripheral red cell concentration, it seems likely that the haematocrit fall is in dogs, as in sheep, due mainly to splenic relaxation. Chlorpromazine will probably be shown to have similar effects in other species in which the blood-storage function of the spleen is relatively important, such as other ruminants, carnivores and horses. In man the effect may well not occur or it may be relatively unimportant, and due mainly to the effect of water shifts; for the spleen is small, its rôle in haematocrit rise after adrenaline is doubtful (Bell *et al.*, 1953), and haematocrit fall does not occur during sleep or even during barbiturate anaesthesia (Reeve, 1946).

The relative importance of central ataractic effect of chlorpromazine, and of its local adrenergic blocking effect in the spleen, cannot be entirely resolved as a result of these limited experiments, but some pointers emerge. Profound haematocrit fall similar to that after chlorpromazine, and like it unaffected by the stimulation of venipuncture, but abolished by splenectomy, occurred with DHE but not with Dibenamine. Although all three were shown to have appreciable anti-catecholamine activity, as measured by inhibition of haematocrit rise, Dibenamine did not cause haematocrit fall, nor was it very effective in preventing haematocrit rise following excitement. DHE has important central effects (Nickerson, 1949; Gilman, 1955), which may be observed with doses lower than those required to produce even minimum peripheral adrenergic blockade (Rosenblueth and Cannon, 1933), whereas Dibenamine is more specific and has little central effect (Nickerson, 1959). Finally, pentobarbitonum, which has virtually no peripheral adrenergic blocking effect but has important central effects, preventing sympatho-adrenal discharge by blocking nervous impulses within the central nervous system (Nickerson, 1959), caused haematocrit fall when administered at a dose sufficient to produce obvious signs of central effect. Chlorpromazine also has important central effects (Courvoiser *et al.*, 1953) which Rothballer (1959) lists as "deactivation of the EEG, lowering of sympathetic tone, lessening of spinal facilitation, . . . as well as its general tranquillizing and soporific qualities". It is therefore suggested that the main responsibility for the haematocrit-lowering effect of chlorpromazine in

sheep rests upon its central effects. How far some of its central effects can be interpreted as central adrenergic-blocking activity is debatable, for Nickerson (1959) deprecates the conclusions of Spector *et al.* (1957), stating that to assume that it can produce effects only by blocking responses to adrenaline and *nor*-adrenaline is "particularly dangerous in the case of a pharmacologically complex agent such as chlorpromazine".

Another tranquillizing agent, mepazine, also had moderate haematocrit-lowering effect and this effect was virtually abolished by splenectomy. It therefore seems that the haematocrit fall is explained by splenic relaxation in this instance also. The manufacturers (Anon., 1956), basing their statements mainly upon the findings of Nieschulz and collaborators, state that it "produces a wide variety of pharmacological effects which collectively inhibit and stabilize the central and peripheral regulating mechanisms of the autonomic nervous system and directly depress the central nervous system". Included in its most important effects are "sedation without hypnosis". In view of the temporary reversal of the haematocrit-lowering effect by excessive stimulation, and the absence of claims that it has adrenergic-blocking activity, the splenic relaxation is probably due to ataractic effect, i.e. increase of the threshold of responsiveness of the cerebral cortex to environmental stimuli.

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